D2 cont'd

Fc domain, an immunoglobulin Fc mutein, a FLAG™ tag, a peptide comprising at least 6 His residues, a leucine zipper, and combinations thereof.

D3

13. (Twice amended) A method of inhibiting RANKL-induced osteoclastogenesis in a patient in need thereof, said method comprising administering to said patient a composition comprising a recombinant soluble RANK polypeptide, wherein said patient suffers from a condition selected from the group consisting of squamous cell carcinoma, lung cancer, prostate cancer, hematologic cancer, head and neck cancer and renal cancer, and further wherein the soluble RANK polypeptide is capable of binding to a RANKL polypeptide that consists of amino acids 1-317 of SEQ ID NO:8 and is selected from the group consisting of:

(a) a polypeptide encoded by a DNA that encodes a protein comprising amino acids 33-196 of SEQ ID NO:2;

(b) a polypeptide encoded by a DNA that is capable of hybridizing to a DNA consisting of the nucleotide sequence shown in SEQ ID NO:1 under stringent conditions, wherein stringent conditions comprise hybridizing at 63°C in 6 x SSC;

(c) a polypeptide that is at least 80% identical in amino acid sequence to amino acids 33-213 of SEQ ID NO:2; and

(d) a polypeptide comprising amino acids 33-213 of SEQ ID NO:2, and

further wherein said composition is administered in amounts sufficient to inhibit RANKL-induced osteoclastogenesis in said patient.

REMARKS

Claims 5, 9, 11, 13, 15, 16, 18, 20, 25 and 26 are presently under consideration in the application. These claims have been rejected in an Office Action dated November 4, 2002 for the reasons that are discussed below. Claims 5, 9 and 13 have been amended as indicated above.

Claim 5 has been amended to replace the phrase "ameliorating the effects of excess bone loss" with "inhibiting RANKL-induced osteoclastogenesis in a patient in need thereof." Support for this amendment is found throughout the specification, for example, at page 5, lines 6-8; page 6, lines 32-35; page 7, lines 9-24; page 8, lines 4-7, 9-11 and 26-30. It is stated at page 7, lines 9-11, for example, that "soluble forms of RANK [are] useful for the inhibition of the RANKL-mediated signal transduction that leads to the differentiation of osteoclast precursors into osteoclasts."

Claim 5 has been amended further to eliminate reference to patients who are "at risk from" one of the listed conditions. Also, part (c) of claim 5 is amended to refer to a RANK polypeptide comprising amino acids 33-213 instead of a polypeptide comprising amino acids 1-213 of SEQ ID NO:2. Support for this amendment is found throughout the

specification, for example, at page 3, lines 4-7. Claim 5 also has been amended to specify that the soluble RANK is "administered in amounts sufficient to inhibit RANKL-induced osteoclastogenesis." This amendment was made to better clarify the intended scope of the claim and is supported throughout the specification, for example, at page 6, line 32 to page 7, line 2 and at page 8, lines 4-6.

Claim 9 has been amended to insert the word "soluble" before the word "RANK." This amendment was made solely to make the language of claim 9 conform with the language used in claims 5 and 11, to which claim 9 is related. This amendment is believed to have no impact whatsoever on the scope of claim 9.

Claim 13 has been amended as described above for claim 5 and these amendments are supported in the specification as described above in connection with claim 5. In addition, claim 13 has been amended to delete the word "therapeutic" from the term "therapeutic composition." Deletion of this adjective is believed to not have any impact on the breadth of this claim. This amendment is supported in the specification, for example, at page 8, lines 9-25.

It is believed that none of the above-described claim amendments constitute the addition of new matter to the application.

In view of the foregoing amendments and the comments below, the examiner is respectfully requested to reconsider the patentability of claims 5, 9, 11, 13, 15, 16, 18, 20, 25 and 26.

Rejections under 35 U.S.C. § 112, Second Paragraph

Claims 5, 9, 11, 13, 15, 16, 18, 20, 25 and 26 stand rejected under 35 U.S.C. § 112, second paragraph, in view of the examiner's assertions that these claims are indefinite.

The examiner believes that claims 5 and 13 are indefinite because it is not clear what is meant by "ameliorating effects of excess bone loss" and because it is not clear what RANK activity is inhibited. These claims have been amended by deleting the phrase "ameliorating effects of excess bone loss" and replacing it with "reducing RANKL-induced osteoclastogenesis." This amendment eliminates the language the examiner found unclear and also specifies what activity is inhibited, i.e., RANKL-induced osteoclastogenesis. Accordingly, the examiner is respectfully requested to remove this ground for rejection.

The examiner has stated that claim 11 is indefinite because it does not further limit claim 9, from which it depends. Claim 9 depends from claim 5, thus the base claim for claim 11 is claim 5. The examiner has asserted similarly that claim 15 is broader than its base claim, which is claim 13. It is believed that these concerns are remedied by the amendments to parts (c) of base claims 5 and 13. As now written, part (c) of claims 5

and 13 reads "a polypeptide that is at least 80% identical in amino acid sequence to a RANK polypeptide comprising amino acids 33-213" rather than "a polypeptide that is at least 80% identical in amino acid sequence to a RANK polypeptide comprising amino acids 1-213." Thus, dependent claims 11 and 15 are narrower than their base claims because they limit the soluble RANK to one member of a Markush group recited in the base claims. Accordingly, the examiner is asked to remove this ground for the rejection of claims 11 and 15.

The examiner has asserted further that claim 5 is indefinite because it is not clear what determines whether a patient is "at risk" for having the conditions recited in the claim. Claim 5 has been amended to delete the "at risk" language, thus this ground for rejection is believed to be moot and its removal is requested.

Claims 9, 16, 18, 20 and 25-26 stand rejected for depending upon an indefinite base claim. Claims 9, 18 and 25 depend directly or indirectly from claim 5. In view of the above-discussed amendments to claim 5, the base claim for claims 9, 18 and 25 should no longer be considered indefinite and this ground for rejection should be withdrawn as applied to these three claims. Claims 16, 20 and 26 depend directly or indirectly from claim 13, which has been amended as discussed above. Thus, the base claim for claims 16, 20 and 26 should no longer be considered indefinite and this ground for rejection should be withdrawn as asserted against these three claims.

In view of the above comments, claims 5, 9, 11, 13, 15, 16, 18, 20, 25 and 26 are now believed to satisfy the requirements of 35 U.S.C. § 112, second paragraph, and the examiner is respectfully requested to withdraw all of the rejections of these claims under this statutory provision.

Rejections under 35 U.S.C. § 112, First Paragraph

Claims 5, 9, 11, 13, 15, 16, 18, 20, 25 and 26 are rejected under 35 U.S.C. § 112, first paragraph, in view of the examiner's position that the specification is enabled for reducing osteoclast differentiation by administering a soluble RANK polypeptide to a patient suffering from squamous cell carcinoma but that it is not enabled for ameliorating effects of excess bone loss in patients at risk for or suffering from bone cancer, multiple myeloma, melanoma, breast cancer, lung cancer, prostate cancer, hemolytic cancer, head and neck cancer and renal cancer.

As noted above, claims 5 and 13, from which claims 11, 13, 15, 16, 18, 20, 25 and 26 directly or indirectly depend, have been amended so that they no longer recite "ameliorating effects of excess bone loss," but instead recite "reducing RANKL-induced osteoclastogenesis." As explained in the specification, increased numbers of osteoclasts and increased osteoclastic bone resorption are observed in patients suffering from the several different types of cancer that are recited in claims 5 and 13 (see, for example,

page 2, lines 7-10 and page 7, lines 13-20). The specification teaches also that RANKL interacts with RANK on osteoclast progenitors to trigger an event that leads to the differentiation of these progenitors into osteoclasts (see, for example, page 7, lines 6-9 and 35-37; page 8, lines 4-6; page 9, line 20 to page 10, line 15). One skilled in the art thus would expect patients with increased osteoclasts to undergo a beneficial reduction in osteoclasts if treated with an agent that blocks the interaction between native RANKL and the native RANK expressed on osteoclast progenitors in the patient. If the examiner believes that this expectation is not credible, he is asked to support his belief by providing specific evidence to document his position.

The examiner has asserted that the specification does not teach how to "make and use" the claimed methods. Applicants believe that this ground for rejection is unjustified, especially in view of the above amendments to the claims. As amended, independent claim 5 instructs those skilled in the art to administer soluble RANK in amounts sufficient to inhibit osteoclasts to a patient who is suffering from bone cancer, multiple myeloma, melanoma or breast cancer. Independent claim 13 recites that patients suffering from squamous cell carcinoma, lung cancer, prostate cancer, hematologic cancer, head and neck cancer or renal cancer are to be treated similarly with soluble RANK. The examiner has not asserted that the specification fails to teach how to make soluble RANK nor has he asserted that the average physician would have any difficulty determining which patients suffer from bone cancer, multiple myeloma, melanoma, breast cancer, squamous cell carcinoma, lung cancer, prostate cancer, hematologic cancer, head and neck cancer or renal cancer. The reasons he has given for rejecting the claims appear to be based in part on concerns that administering soluble RANK would not "ameliorate effects of excess bone loss" in the treated patients. However, claims 5 and 13, as amended, no longer state that the soluble RANK will "ameliorate effects of excess bone loss," a phrase that the examiner found unclear, but rather that it will reduce the numbers of osteoclasts in the patient. The examiner has provided no evidence that casts doubt on the expectation that patients treated with soluble RANK will experience reduced osteoclasts. If he has reason to believe that osteoclasts would not become reduced in these patients, he is asked to provide documentation in support of this position.

The examiner also has expressed concern that one skilled in the art would have to engage in "undue experimentation" to determine effective compositions and doses. However, the determination of dose and frequency of administration is generally considered to be a routine matter for those skilled in the art (see the specification at page 8, lines 22-25 and lines 30-33). The examiner has presented no evidence showing that developing a dosing regimen for soluble RANK would be an exception to this general rule. He is respectfully requested to provide such evidence or alternatively to acknowledge that the application provides adequate support for dosing regimens.

Furthermore, the specification teaches compositions suitable for administration to patients (page 8, lines 8-22), and in any case making such formulations usually are considered to be routine for those skilled in the art. In view of these comments, the examiner is asked to remove this ground for the rejection of claims 5, 9, 11, 13, 15, 16, 18, 20, 25 and 26.

As explained above, the present claims are believed to be fully enabled by the specification, particularly in view of the amendments to independent claims 5 and 13. The attached Exhibits A-D add further credence to the specification's assertions that osteoclasts will become reduced in patients treated with agents that block the interaction of RANK and RANKL. Although these exhibits were published after the filing date to which the present claims are entitled, they nonetheless reinforce the applicants' position that the methods claimed herein are enabled by the specification. Exhibit A, Morony et al.[1], presents data showing that the number of osteoclasts became reduced in a mouse breast tumor model following treatment with the RANKL inhibitor OPG. OPG, like soluble RANK, prevents RANKL from binding to RANK on osteoclast progenitors. Oyajobi et al.[2] (Exhibit B) presents experiments using an animal model for multiple myeloma. When these animals were given RANK:Fc, the number of detectable osteoclasts was reduced and tumor burden appeared reduced also. Pearse et al.[3] (Exhibit C) shows similarly that in the SCID-hu murine model of human myeloma, administering RANK:Fc resulted in fewer osteoclasts (see, for example, Figure 3E in Pearse et al.). Exhibit D (Zhang et al. [4]) teaches that the osteoclastogenesis induced by prostate cancer cells can be abrogated in an animal model by administering OPG. These examples from the literature reinforce applicants' contention that osteoclast levels will become reduced if soluble RANK is administered to an individual who suffers from one of the cancers recited in claims 5 and 13.

The examiner has asserted also that it is unpredictable what diseases could be effectively treated using a RANK polypeptide. However, those skilled in the art would have no need to make such a determination because the claims at issue recite which diseases are to be treated.

The examiner also has rejected claims 13, 15, 16, 18, 25 and 26 in view of the phrase "therapeutically sufficient amount of a composition comprising a RANK polypeptide." The basis for this rejection is unclear since this phrase does not appear in any of claims 13, 15, 16, 18, 25 or 26. However, the term "therapeutic composition" appears in claim 13. The applicants believe that the inclusion of the term "therapeutic" in this claim is fully justified in light of the disclosure of the application. The application

[1] Morony et al., *J Bone Min Res* 17:S147, #1092 (2002)
[2] Oyajobi et al., *J Bone Min Res* 15:S176 (2000)
[3] Pearse et al., *Proc Natl Acad Sci USA* 98(20):11581-86 (2001). Here, RANKL is called "TRANCE."
[4] Thomas et al., *Endocrinol* 140:4451-58 (1999)

teaches that patients suffering from the cancers recited in claim 13 are characterized by bone loss due to excess osteoclasts and that osteoclast numbers can be reduced in such patients by administering soluble RANK (see, for example, page 2, lines 5-12 and page 7, lines 11-24). Nonetheless, to expedite prosecution of this application, claim 13 has been amended to delete the word "therapeutic." The amendment to claim 13 also is applicable to claims 15, 16 and 26 due to their direct or indirect dependency from claim 13. Claims 18 and 25 do not depend directly or indirectly from any claim that contains the term "therapeutic," thus this ground for rejection appears to be misapplied to these two claims. In view of these comments and the amendment to claim 13, the examiner is kindly asked to remove this ground for rejection of claims 13, 15, 16, 18, 25 and 26.

CONCLUSIONS

Claims 5, 9, 11, 13, 15, 16, 18, 20, 25 and 26 remain under consideration and have been rejected under 35 U.S.C. § 112, first and second paragraphs. In view of the amendments and remarks set forth above, these claims are now believed to be in condition for allowance and notification to this effect is respectfully requested. If further areas of concern remain in the application, the examiner is asked to contact the undersigned at her direct dial number given below.

Respectfully submitted,

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22932

CERTIFICATE OF MAILING

I hereby certify that this Amendment and Response to Paper No. 12 is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Washington, DC 20231, on the date indicated below.

Date: April 3, 2003

D. F. Lindholm

APPENDIX
TO AMENDMENT and RESPONSE TO PAPER NO. 12
(marked-up version of paragraphs amended in the attached Amendment)

Claims:

5. (Three times amended) A method of [ameliorating effects of excess bone loss] inhibiting RANKL-induced osteoclastogenesis in a patient in need thereof, comprising administering to said patient a soluble RANK polypeptide composition [to an individual at risk for excess bone loss], wherein said [individual] patient [is at risk from or] suffers from a condition selected from the group consisting of bone cancer, multiple myeloma, melanoma and breast cancer, and further wherein the soluble RANK polypeptide is capable of binding to a RANKL polypeptide that consists of amino acids 1-317 of SEQ ID NO:8 and is selected from the group consisting of:

(a) a polypeptide encoded by a DNA that encodes a protein comprising amino acids 33-196 of SEQ ID NO:2;

(b) a polypeptide encoded by a DNA that is capable of hybridizing to a DNA consisting of the nucleotide sequence shown in SEQ ID NO:1 under stringent conditions, wherein stringent conditions comprise hybridizing at 63°C in 6 x SSC;

(c) a polypeptide that is at least 80% identical in amino acid sequence to a RANK polypeptide comprising amino acids [1]33-213 of SEQ ID NO:2; and

(d) a polypeptide comprising amino acids 33-213 of SEQ ID NO:2, and

further wherein said composition is administered in amounts sufficient to inhibit RANKL-induced osteoclastogenesis in said patient.

9. (Twice amended) The method of claim 5, wherein the soluble RANK further comprises a polypeptide selected from the group consisting of an immunoglobulin Fc domain, an immunoglobulin Fc mutein, a FLAG™ tag, a peptide comprising at least 6 His residues, a leucine zipper, and combinations thereof.

13. (Twice amended) A method of [ameliorating the effects of excess bone loss] inhibiting RANKL-induced osteoclastogenesis in [comprising administering to] a patient in need thereof, said method comprising administering to said patient a [therapeutic] composition comprising a recombinant soluble RANK polypeptide, wherein said patient suffers from a condition selected from the group consisting of squamous cell carcinoma, lung cancer, prostate cancer, hematologic cancer, head and neck cancer and renal cancer, and further wherein the soluble RANK polypeptide is capable of binding to

a RANKL polypeptide that consists of amino acids 1-317 of SEQ ID NO:8 and is selected from the group consisting of:

(a) a polypeptide encoded by a DNA that encodes a protein comprising amino acids 33-196 of SEQ ID NO:2;

(b) a polypeptide encoded by a DNA that is capable of hybridizing to a DNA consisting of the nucleotide sequence shown in SEQ ID NO:1 under stringent conditions, wherein stringent conditions comprise hybridizing at 63°C in 6 x SSC;

(c) a polypeptide that is at least 80% identical in amino acid sequence to amino acids [1]33-213 of SEQ ID NO:2; and

(d) a polypeptide comprising amino acids 33-213 of SEQ ID NO:2, and

further wherein said composition is administered in amounts sufficient to inhibit RANKL-induced osteoclastogenesis in said patient.

1091

Monitoring Progression of Breast Cancer Cells in Bone/Bone Marrow by Optical Imaging: Bisphosphonates do not Suppress Tumor Growth Rate and Tumor Burden. G. van der Pluijm[1], B. Sijmons*[1], I. Que*[1], J. Buijs*[1], M. Cecchini*[2], C. Löwik[1], S. Papapoulos[1]. [1]Endocrinology, Leiden University Medical Center, Leiden, Netherlands, [2]Urology, University Hospital, Berne, Switzerland.

Bone metastases are common in patients with breast and prostate cancer and cause considerable morbidity and deterioration of the quality of life. Bisphosphonate (Bp) treatment reduces skeletal-related events by inhibiting tumor-induced osteolysis. In addition, local anti-tumor effects of Bps have been reported in vitro and in an in vivo model of metastatic bone disease. In the latter model, however, evaluation of tumor burden was assessed by measuring lytic bone lesion areas (radiographs) and histomorphometric analyses of tumor areas within the bone collar. In most studies, therefore, the suggested reduction in tumor burden is based primarily on the osteoprotective properties of Bps. Recently, we established an extremely sensitive, non invasive, optical imaging method to detect, monitor and quantify luciferase transfected tumor cells (MDA-MB-231/luc+) in vivo. Optical imaging also enables continuous monitoring in the same animal of growth kinetics for each metastatic site and allows the measurement of the overall tumor burden by photon counting (Am. J. Pathol. 160:1143,2002). We applied this model to determine the effects of different Bps on the local progression of human breast cancer cells in the bone/bone marrow microenvironment. One week after intraosseous injection of MDA-MB-231/luc+ cells into the tibiae of nude mice, the animals were treated with various high doses of bisphosphonates (clodronate, pamidronate, olpadronate) previously shown to completely inhibit osteoclastic bone resorption. Bps were either given continuously (mini-osmotic pumps) or by daily s.c. injections for 28 days. As expected, all tested Bps significantly inhibited cancer-induced bone destruction measured by osteolytic bone areas. In contrast, however, the tested Bps did not inhibit tumor progression when quantified by optical imaging (photon counting after 1-2-3-4 weeks) confirmed by histomorphometrical analysis at the end of each experiment. Our data, therefore, provide no evidence of an anti-tumor effect by Bps despite the observed protection of bone integrity.

1092

OPG Inhibits the Progression of Bone Destruction and Skeletal Tumor Burden in Mice With Established Osteolytic MDA-231 Breast Cancer Metastases. S. Morony, K. Warmington*, H. Tan, V. Shalhoub, G. Chow*, C. R. Dunstan, D. L. Lacey, P. J. Kostenuik. Metabolic Disorders, Amgen Inc., Thousand Oaks, CA, USA.

OPG, a RANKL decoy receptor, inhibits osteoclast formation and activation by preventing RANKL-RANK interactions on osteoclasts and their precursors. We previously demonstrated that OPG treatment started at the time of MDA-231 cell inoculation inhibited tumor-associated osteolysis and reduced skeletal tumor burden (Morony et al, Cancer Res 61:4432, 2001). The present study tested whether OPG treatment would prevent the progression of osteolysis and skeletal tumor burden after MDA-231 lesions were already established in bone. Nude female mice were inoculated in the left ventricle with MDA-231 tumor cells. Three weeks later, digitized radiographs of all mice were analyzed to quantify the area of osteolytic lesions. Mice without radiographic evidence of osteolysis were excluded from the study. The remaining mice were allocated to groups that had similar average baseline areas of osteolysis. At baseline, group means for area of osteolysis ranged from 0.88 to 0.99 mm2/mouse (n=13-15/group). One group of tumor-bearing mice was sacrificed prior to treatment for the histological determination of baseline skeletal tumor burden (1.87 ± 0.6 mm2/mouse). Mice were treated with PBS (vehicle) or with OPG (3 mg/kg, SC) on days 23, 24, 27, 29, and 31 post inoculation, and were sacrificed on day 31. The radiographic area of osteolysis increased by 400% during PBS treatment (3.31 ± 0.44 mm2/mouse, p<0.01) and decreased by a non-significant 19% (0.81 ± 0.19 mm2/mouse) during OPG treatment. Histologically, skeletal tumor burden increased 210% (5.79 ± 1.32 mm2/mouse, p<0.01) during PBS treatment, while the increase during OPG treatment was a non-significant 32% (2.47 ± 0.82 mm2/mouse). The differences in osteolysis and skeletal tumor burden between OPG and PBS treated groups were statistically significant (p<0.01). OPG treatment also reduced the number of osteoclasts per mm2 of skeletal tumor area by 50% vs. PBS treated mice (p<0.01). The inhibitory effect of OPG on skeletal tumor burden might be related to the inhibition of bone resorption. A direct effect of OPG on tumor cells was not supported by cell culture studies. Neither OPG nor RANKL had any effect on MDA-231 cell number during 48 h of culture with various concentrations of fetal bovine serum. These data support the hypothesis that OPG indirectly inhibits MDA-231 tumor burden in bone via its potent antiresorptive effects. The ability of OPG to suppress the bone destruction and the skeletal tumor burden associated with established osteolytic lesions underscores the potential utility of OPG as a therapeutic option for the treatment of metastatic bone disease.

1093

Gene Transfer of Osteoprotegerin-Fc Inhibits Osteolysis & Progression in a Murine Model of Multiple Myeloma. P. I. Russell*[1], D. Chen*[2], S. M. Greiner*[1], J. Ludvigson*[1], S. Khosla[1], B. L. Riggs[1]. [1]Mayo Clinic, Rochester, MN, USA, [2]Amgen Inc., Thousand Oaks, CA, USA.

Multiple myeloma has major adverse skeletal effects, including bone pain, pathologic fractures, and hypercalcemia, that are only transiently and incompletely reversed by currently available therapy. Osteoprotegerin (OPG) potently inhibits osteoclast differentiation and activation, but, if used therapeutically, it or the more prolonged-acting OPG-Fc-fusion protein analog require repeated subcutaneous injections. Gene therapy has shown promise in the treatment of various malignancies. Thus, as proof-of-principle for antiresorptive gene therapy of osteolytic bone disease, we evaluated the effect of *ex vivo* OPG-Fc gene transfer in a murine model of multiple myeloma. In this model, injection of human ARH-77 cells into 6-week-old SCID mice results in severe osteolytic lesions. In the therapeutic group, the injected ARH-77 cells were stably transduced with the OPG-Fc-fusion protein gene using a replication-incompetent lentiviral vector (OPG-Fc, n=18). Effects were compared with those in positive control mice (PC, n=19) injected with the same number of ARH-77 cells that were similarly transduced with a non-expressing control vector, and with those in negative control mice (NC, n=20) that did not receive ARH-77 cells. The mean ± SEM level of serum OPG-Fc levels obtained at sacrifice was undetectable (<0.02 ng/mL) in the control groups but was 966 ± 239 ng/mL at sacrifice (P<0.001) in the OPG-Fc expressing group. None of the NC mice developed any disease manifestations. The median time for onset of partial paraplegia was 22 days in the PC as compared to 30 days in the OPG-Fc mice (P<0.001), and complete paraplegia occurred in 84% of PC as compared to 39% of OPG-Fc mice (P<0.001). Total body BMD was greater at 4 weeks in the OPG-Fc mice (0.0404 ± 0.0004 g/cm^2) than in the PC and NC mice (0.0394 ± 0.0003, P=0.05 and 0.0393 ± 0.0003, P=0.04, respectively). Osteolytic radiographic lesions developed in 78% of PC mice, but in only 17% of the OPG-Fc expressing mice (P<0.001). There were similar differences when osteolytic lesions were quantified by number, diameter, and the sum of diameters (all P<0.001). Finally, median survival time was 32 days compared with 37 days in the PC and OPG-Fc groups, respectively (P=0.005). In conclusion, our finding that *ex vivo* OPG-Fc gene transfer dramatically attenuates the skeletal manifestations of murine multiple myeloma suggests that therapeutic gene transfer using this or other genes offers considerable promise as a novel therapy for neoplastic bone disorders, either alone or in combination with conventional therapies.

Disclosures: P. Doran, David Chen, Amgen Inc. 3.

1094

Bisphosphonates Induce Apoptotic Prostate Cancer Cell Death in Vitro. J. C. Dumon*[1], F. Journé*[1], N. Khedoumi*[1], L. Lagneaux*[2], J. J. Body[1]. [1]Lab. of Endocrinology and Breast Cancer Research, Inst. J. Bordet, Free University of Brussels, Brussels, Belgium, [2]Lab. of Hematology, Inst. J. Bordet, Free University of Brussels, Brussels, Belgium.

Bone tissue is the most common site of distant metastases in breast and prostate cancers. Bisphosphonates have been shown to reduce the morbidity rate of metastatic bone disease from breast cancer and, quite recently, also from prostate cancer. These effects are attributed to their powerful inhibitory activity on osteoclast-mediated bone resorption. We have shown that bisphosphonates also induce human breast cancer cell death in vitro (Fromigué et al, JBMR 2000). We have now investigated their effects on the survival, growth and death of prostate cancer cells (PC3) in vitro after 1, 2, 4 and 6 days of incubation. We tested four bisphosphonates at doses ranging from 10-8 to 10-4M: clodronate (Clod), ibandronate (Iban), pamidronate (Pam), and zoledronate (Zole). The inhibitory effects on PC3 cells viability (evaluated by the MTT test) were maximal at 10-4M and this concentration was used for further experiments. The MTT results are summarised below as % of control cell survival (mean±SEM). Total DNA content was also decreased at day 6: to 46±6% for Clod, to 31±3% for Iban, to 46±2% for Pam, and to 4±0.2% for Zoledronate. FACS experiments showed that, at day 1, there was already a marked decrease (except for clodronate) in the number of cells in "Synthesis and Mitosis" phases (decreases of 24% for Iban, 57% for Pam, and 43% for Zole). At day 4, according to the bisphosphonate, 50% to 90% of the cell population was apoptotic. In conclusion, bisphosphonates caused an early inhibition of prostate cancer cell proliferation followed by cell death, essentially through apoptosis. Zoledronate was the most potent compound with an almost complete cell death at day 6. Such effects could contribute to the beneficial role of bisphosphonates in the treatment and the prevention of prostate cancer-induced bone disease.

	Clod	Iban	Pam	Zole
Day 1	100±5	95±5	85±12	89±5
Day 2	101±12	90±3	67±5	71±7
Day 4	80±5	75±12	56±15	71±6
Day 6	77±7	70±6	49±9	16±6

J. BONE MINERAL RES. 2000 15:S176

A Soluble Murine Receptor Activator of NF-κB-Human Immunoglobulin Fusion Protein (RANK.Fc) Inhibits Bone Resorption In a Murine Model of Human Multiple Myeloma Bone Disease

Presentation Time: Sunday, 4:45 p.m. - 5:00 p.m.

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Presentation Number: 1151

Conventional chemotherapy regimens for multiple myeloma show little or no efficacy in ameliorating the lytic bone lesions characteristic of this malignancy. Although the use of bisphosphonates is being investigated for this purpose, the beneficial effects of bisphosphonates in myeloma are modest. Therefore, effective new therapeutic agents that inhibit osteoclastic bone resorption are desirable. RANK, OPG and their cognate ligand (RANKL) regulate osteoclastogenesis and bone resorption in vivo. Whereas RANK mediates signals essential for osteoclast formation and activation, OPG acts solely as a decoy receptor. OPG, but not RANK, also binds TRAIL, a ubiquitously expressed cytokine that is cytotoxic to myeloma cells. We have previously demonstrated the efficacy of RANK.Fc (a genetically-engineered soluble form of murine RANK fused to the Fc domain of human IgG) in inhibiting bone resorption in vitro and in vivo. Importantly, unlike OPG, RANK.Fc does not interact with TRAIL and thus cannot abrogate any potential beneficial effect of TRAIL in myeloma. In this study, we investigated the utility of RANK.Fc as a treatment option in multiple myeloma using an animal model of human myeloma bone disease in which murine 5TGM1 myeloma cells are inoculated by intravenous transfer into C57BL/KaLwRij or nude (bg-nu-xid) mice. In this model, tumor-bearing mice exhibit features similar to those associated with human multiple myeloma including bone destruction. RANK.Fc was administered as daily (100μg) subcutaneous injections for 28 days. Static histomorphometric analyses were carried out on sections of long bones and vertebrae and soft tissue sections were assessed for tumor infiltration. There was no overt evidence of toxicity in either tumor-bearing mice or non tumor-bearing (control) mice that received the chimeric fusion protein. Treatment of tumor-bearing mice with RANK.Fc resulted in a profound inhibition of osteoclastic activity as evident by the significantly reduced number of TRAP-staining multinucleated osteoclasts, accompanied by markedly increased bone density visible radiologically at the metaphyses of long bones. Histomorphometry revealed a marked increase in bone trabeculae in both distal femoral and proximal tibial metaphyses comparable to that in non tumor-bearing mice treated with RANK.Fc. Furthermore, there was a concomitant reduction in tumor volume in the same regions in RANK.Fc-treated tumor-bearing mice when compared with vehicle-treated tumor-bearing mice. We have not found a beneficial effect of bisphosphonates on tumor bulk (Blood 93: 1697, 1999). Our data thus indicate a key role for RANKL/RANK interactions in the pathogenesis of osteolytic lesions in multiple myeloma and we conclude that targeted disruption of these interactions with RANK.Fc offers a potential and novel therapeutic intervention for the treatment of myeloma-associated bone destruction, and possibly myeloma itself.

Multiple myeloma disrupts the TRANCE/osteoprotegerin cytokine axis to trigger bone destruction and promote tumor progression

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Bone destruction, caused by aberrant production and activation of osteoclasts, is a prominent feature of multiple myeloma. We demonstrate that myeloma stimulates osteoclastogenesis by triggering a coordinated increase in the tumor necrosis factor-related activation-induced cytokine (TRANCE) and decrease in its decoy receptor, osteoprotegerin (OPG). Immunohistochemistry and *in situ* hybridization studies of bone marrow specimens indicate that *in vivo*, deregulation of the TRANCE–OPG cytokine axis occurs in myeloma, but not in the limited plasma cell disorder monoclonal gammopathy of unknown significance or in nonmyeloma hematologic malignancies. In coculture, myeloma cell lines stimulate expression of TRANCE and inhibit expression of OPG by stromal cells. Osteoclastogenesis, the functional consequence of increased TRANCE expression, is counteracted by addition of a recombinant TRANCE inhibitor, RANK-Fc, to marrow/myeloma cocultures. Myeloma–stroma interaction also has been postulated to support progression of the malignant clone. In the SCID-hu murine model of human myeloma, administration of RANK-Fc both prevents myeloma-induced bone destruction and interferes with myeloma progression. Our data identify TRANCE and OPG as key cytokines whose deregulation promotes bone destruction and supports myeloma growth.

Multiple myeloma (MM) is a B cell neoplasm characterized by clonal expansion of plasma cells. MM typically involves bone and marrow, suggesting dependence on these microenvironments for survival and proliferation (1–3). In addition, MM stimulates production of activated osteoclasts (OCs) from monocytic precursors, leading to severe osteoporosis and bone destruction in most patients (4–6). A variety of MM-produced cytokines, including IL-1β, IL-6, IL-11, tumor necrosis factor (TNF) α, and lymphotoxin-α, can stimulate osteoclastogenesis *in vitro*. However, production of these cytokines in patients with MM is heterogeneous, thus none has been shown to be the common mediator of MM-induced osteoclastogenesis (6).

Evidence from gene-deleted and transgenic mice indicates that generation of activated OCs from monocytic precursors is controlled by coordinate expression of the TNF-related activation-induced cytokine (TRANCE; OPGL, RANKL, ODF, TNFSF11) and its decoy receptor osteoprotegerin (OPG; OCIF, TNFRSF11b) (7–11). TRANCE is expressed on the surface of activated T cells, marrow stromal cells, and osteoblasts as a 45-kDa transmembrane protein and, in solution, as a 31-kDa product of metalloproteinase cleavage (13–17). TRANCE triggers development and activation of OCs by binding to its functional receptor, RANK (TNFRSF11a), expressed on OCs and their precursors as an integral membrane protein (17). OPG is secreted by stromal cells as a soluble 110-kDa disulfide-linked homodimer (11, 12). Mice that lack either TRANCE or RANK, or that overexpress OPG, develop osteopetrosis because of decreased OC activity (7–11). Conversely, mice that lack OPG exhibit profound osteoporosis as a consequence of unopposed TRANCE activity (9). In addition, mice deficient in either TRANCE or receptor activator of NF-κB (RANK) exhibit defective lymph node organogenesis and early B cell development (7, 10). However, the role of TRANCE and RANK in plasma cell differentiation and survival has not been evaluated.

We present evidence that MM disrupts the balance between TRANCE and its inhibitor, OPG. In addition, we show that TRANCE inhibition prevents MM-induced bone destruction and interferes with MM progression in two murine models of human MM. These findings identify TRANCE and OPG as key factors whose deregulation promotes bone destruction and supports MM growth.

Materials and Methods

Human Samples, Experimental Animals, and Reagents. This study was performed in accordance with federal and institutional guidelines for human subject and animal research. Generation of SCID/ARH-77 and SCID-hu-MM mice has been described (18–21). To generate SCID-hu-MM mice, 5×10^6 mononuclear cells from marrow of patients with MM-associated bone disease were injected into the xenograft. TRANCE-deficient mice were generated as described (8). Human transforming growth factor (TGF) β1 was obtained from R & D Systems. PGE_2 was obtained from Sigma. Human CSF-1 was the kind gift of R. Stanley (Albert Einstein Medical College, Bronx, NY). RANK-Fc, a fusion of murine RANK (amino acids 22–201) with the Fc region of human IgG_1 that can block both murine and human TRANCE activity, was prepared in *Sf9* cells (PharMingen) (15). TRANCE and OPG were prepared in 293T cells (15).

Histology. Bone marrow from 14 MM patients with bone disease and 13 non-MM patients [five normal, two non-Hodgkin's lymphoma (NHL), one Hodgkin's disease, one chronic lymphocytic leukemia, one chronic myelogenous leukemia, and three monoclonal gammopathy of unknown significance (MGUS) without evidence of progression to MM during 18–48 months follow-up] was evaluated for TRANCE and OPG expression. Concurrent samples from two biopsy sites were available for three MM and three non-MM patients (one NHL, one Hodgkin's disease, one normal). The MM and non-MM groups

Abbreviations: Dpd, deoxypyridinoline crosslinks; MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance; OAF, osteoclast activating factor; OC, osteoclast; OPG, osteoprotegerin; SCID, severe combined immunodeficiency; TRANCE, TNF-related activation-induced cytokine; TRAP, tartrate-resistant acid phosphatase; RANK, receptor activator of NF-κB; TNF, tumor necrosis factor; TGF, transforming growth factor; NHL, non-Hodgkin's lymphoma; RT, reverse transcription.

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did not differ significantly in distribution of age or sex. TRANCE expression was also evaluated in five plasmacytomas arising from bone: four from patients with concurrent MM and one from a patient with a solitary plasmacytoma.

Immunohistochemistry. Four-micrometer sections of formalin-fixed, decalcified, bone marrow or formalin-fixed plasmacytoma were heated in an 80°C oven for 60 min, deparaffinized, rehydrated, and treated with 1.5% hydrogen peroxide for 10 min. Antigen retrieval was accomplished by pretreatment for 10 min with either microwave (OPG) or 0.5% pepsin (TRANCE). Three anti-TRANCE antibodies, MAB626 (R & D Systems), IMG-133 (Imgenex), and sc-7627 (Santa Cruz Biotechnology), gave similar staining patterns at 1:100, although the goat polyclonal antibody (sc-7627) produced background staining that was not seen with either monoclonal antibody. Two anti-OPG antibodies, IMG-103 (Imgenex, San Diego, 1:300 dilution) and sc-8468 (Santa Cruz Biotechnology, 1:1,000), were used with similar staining. Staining for either TRANCE or OPG could be blocked by incubation with specific peptide. Sections incubated with rabbit or murine primary antibodies were blocked with ChemMate blocking antibodies (Ventana Medical Systems, Tuscon, AZ) and stained by using the ChemMate secondary detection kit-peroxidase/diaminobenzidine. Sections incubated with goat primary antibodies were blocked with normal goat serum (Santa Cruz Biotechnology) and stained by using the goat ABC staining system (Santa Cruz Biotechnology). Consistent results were obtained for slides from each individual stained on different days and also for marrow samples taken from a second site. Normal tonsil served as control for TRANCE staining. Vascular staining, which was consistent among all samples, served as control for OPG staining.

***In Situ* Hybridization.** Bone marrow from nine MM and five non-MM (one MGUS, two NHL, two normal) patients was processed with [α-^{33}P]UTP-labeled sense and antisense riboprobes as described (22). The *Bam*HI/*Eco*RI fragment of human TRANCE cDNA (nucleotides 350–930) was used as probe. Sense riboprobes gave no signal above background.

Tartrate-Resistant Acid Phosphatase (TRAP) Cytochemistry. Xenografts were harvested from SCID-hu mice, fixed in formalin, decalcified with EDTA, and embedded in paraffin. Five micrometer deparaffinized sections were immersed in acetone and stained for TRAP according to the manufacturer's instructions (Sigma). TRAP-positive multinucleated giant cells (OCs) in four nonoverlapping mm^2 areas were counted.

Cell Culture. MG63, U2OS, ARH-77, U266, H929, and RPMI 8226 cells were obtained from American Type Culture Collection. ARP-1 cells were the generous gift of J. Hardin (University of Arkansas for Medical Sciences). Primary murine stromal cells were isolated from the calvaria of newborn 129 SV mice as described (23). Conditioned media, generated over 4 days by coculture of MM cell lines with primary murine stromal cells, was concentrated 4-fold by using Centriprep 10 columns (Millipore) and used at a dilution of 1:4. *In vitro* osteoclastogenesis was performed as described (24). Briefly, murine marrow was cultured with CSF-1 (50 ng/ml), PGE_2 (1 μM), and TRANCE (1 μg/ml). In some experiments, TRANCE was replaced by primary murine stromal cells isolated from wild-type or TRANCE-deficient mice cocultured with one of three human MM cell lines. TRAP activity was analyzed according to the manufacturer's instructions (Sigma).

Reverse Transcription (RT)-PCR. mRNA was prepared by using Trizol (GIBCO) and OLIGOTEX (Qiagen, Chatsworth, CA). cDNA was generated by using Moloney murine leukemia virus RT and oligo(dT) (Amersham Pharmacia). PCR was performed for 40 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C) by using the following primer pairs to detect murine TRANCE and actin, respectively: 5′-ATCAGAAGACAGCACTCAC-3′/5′-TTCGTGCTCCCTCCTTTCAT-3′ and 5′-GTGACGAGGCCCAGAGCAAGAG-3′/5′-AGGGGCCGGACTCATCGTACTC-3′.

PCR was performed for 35 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C) by using the following primer pairs to detect human OPG and actin, respectively: 5′-GTGGTGCAAGCTGGAACCCCAG-3′/5′-AGGCCCTTCAAGGTGTCTTGGTC-3′ and 5′-CCTTCCTGGGCATGGAGTCCT-3′/5′-GGAGCAATGATCTTGATCTTC-3′.

Northern Analysis. RNA was prepared by using Trizol, separated by agarose gel electrophoresis in formaldehyde (20 μg total RNA/lane) and blotted to Hybond N+ (Amersham Pharmacia). Hybridization was performed by using an [α-^{32}P]UTP-labeled antisense riboprobe generated by using T7 polymerase (Ambion) and a PCR fragment of human OPG (nucleotides 478–1,124) linked to the T7 promoter.

ELISA. Titers of MM paraprotein were determined as described (21) by using Immulon 2HB microtiter plates (Dynex Technologies, Chantilly, VA) and antibodies purchased from Southern Biotechnology Associates. Urinary crosslinked deoxypyridinoline (Dpd) was assayed according to the manufacturer's instructions (Metra Biosystems, Mountain View, CA). To compensate for diurnal variation in Dpd excretion, urine was collected at the same time on consecutive days and assayed separately for Dpd and creatinine (Sigma); the measured Dpd (nmol)/creatinine (mmol) was then averaged (25).

Results

Deregulation of TRANCE and OPG in Marrow of Patients with MM. Bone marrow biopsies from MM and non-MM patients were evaluated for TRANCE and OPG expression by using riboprobes specific for TRANCE and antibodies specific for TRANCE and OPG (Fig. 1*A*). Both immunohistochemistry and *in situ* hybridization reveal foci of increased TRANCE expression in MM marrow samples but little TRANCE expression in most non-MM samples. Within MM-infiltrated marrows, TRANCE expression is increased in areas that also possess normal marrow elements. In areas of marrow completely replaced by MM, almost all cells express light chain, but TRANCE-positive cells are extremely rare. Similarly, TRANCE was not expressed by Ig-positive cells in any of the five plasmacytomas of bone evaluated, although TRANCE was expressed by rare cells within the plasmacytoma and by lining cells found at the periphery of the tumor in several specimens. Comparison with sections of bone marrow stained for other markers suggests that CD3+, CD30+ activated T cells are the major TRANCE-positive cells in non-MM bone marrow and are a subset of the TRANCE-positive cells in MM bone marrow. In MM, other stromal elements, but not CD34+ endothelial cells, also express TRANCE. Consistent with these observations, we did not find TRANCE expression by the MM cell lines ARP-1, U266, RPMI 8226, H929, and ARH-77, as assessed by RANK-Fc binding or by RT-PCR (data not shown). Together, these results indicate that TRANCE expression is increased in MM-infiltrated marrow by the interaction of malignant plasma cells with activated T cells and stromal cells.

In addition to increased expression of TRANCE, MM-infiltrated marrows exhibit decreased expression of the TRANCE inhibitor, OPG. In normal marrow, OPG-specific antibodies show intense staining of megakaryocytes, stromal cells, and vessels. In marrow infiltrated by MM, vascular staining is evident, but staining of megakaryocytes and stromal cells is markedly decreased. The pattern of increased TRANCE expres-

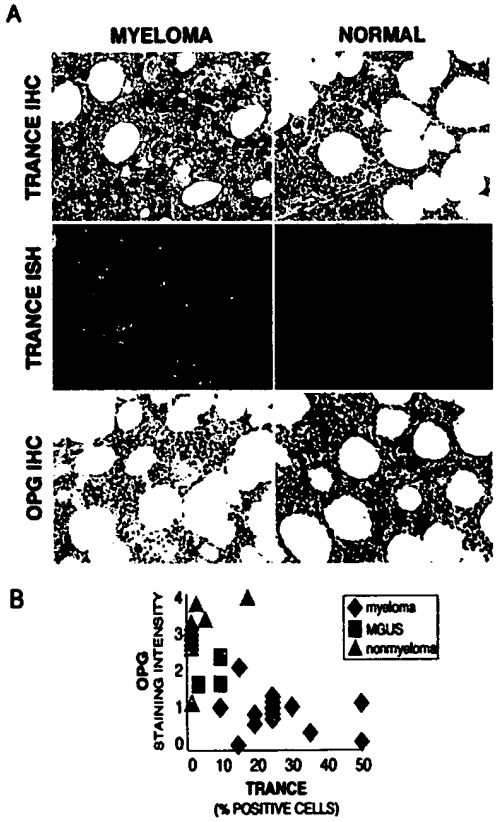

Fig. 1. Marrow infiltration by MM is associated with increased TRANCE and decreased OPG expression. (*A*) Representative images of MM and normal bone marrow following immunohistochemistry (IHC, light field ×600) and *in situ* hybridization (ISH, dark field ×200). Intense staining by a TRANCE-specific antibody and increased hybridization by a TRANCE-specific riboprobe are seen in MM, but not normal marrow. In contrast, staining by OPG-specific antibody is dramatically decreased in MM compared with normal marrow. (*B*) Graphic display of TRANCE and OPG protein expression. Bone marrow biopsies from 14 MM and 13 non-MM patients (three MGUS, two NHL, one chronic lymphocytic leukemia, one chronic myelogenous leukemia, one Hodgkin's disease, and five normal) were evaluated for expression of TRANCE and OPG by IHC and independently graded by two investigators without knowledge of the diagnosis. TRANCE staining was scored on a scale from 0 to 100 based on the percentage of positive cells in foci of increased staining. The mean scores for TRANCE expression in MM and non-MM samples were 26 and 4.5, respectively ($P < 0.001$, Mann–Whitney test). OPG expression was graded on a 0 (none) to 4 (heavy) scale based on the number and intensity of cells stained. The mean scores for OPG expression in MM and non-MM samples were 1.0 and 2.6, respectively ($P < 0.001$, Mann–Whitney test).

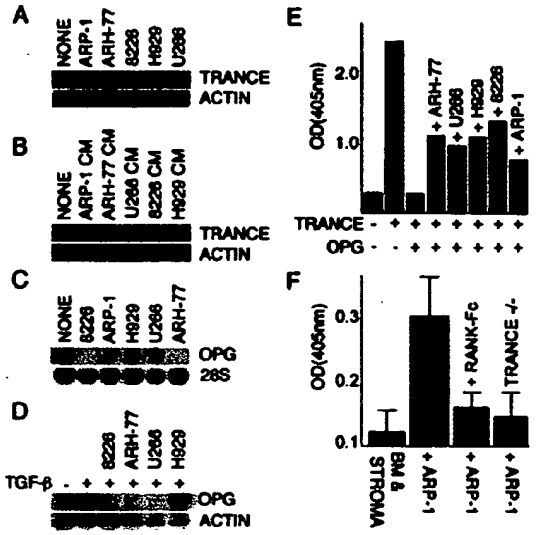

Fig. 2. MM triggers OC development through deregulation of the TRANCE–OPG cytokine axis. (*A*) MM stimulates stromal expression of TRANCE. Primary murine stromal cells were cultured for 4 days without (lane 1) or with (lanes 2–6) each of five MM cell lines (10^5 cells/ml). Stromal cell mRNA was isolated and TRANCE expression was determined by RT-PCR by using primers specific for murine TRANCE and β-actin. (*B*) Media conditioned by MM–stroma coculture stimulates stromal expression of TRANCE. Primary murine stromal cells were cultured for 4 days without (lane 1) or with (lanes 2–6) media condition by the coculture of stroma with each of five MM cell lines. Stromal TRANCE mRNA expression was determined as above. (*C*) MM inhibits stromal expression of OPG. MG63 cells were cultured without (lane 1) or with (lanes 2–6) five MM cell lines (10^5/ml). After 4 days, stromal RNA was isolated and subjected to Northern analysis by using ribosomal and OPG-specific riboprobes. (*D*) MM inhibits TGF-β-induced expression of OPG. U2OS cells were stimulated with TGF-β1 (200 nM) in the absence (lane 2) or presence (lanes 3–6) of four MM cell lines (10^5/ml). After 4 days, stromal mRNA was isolated and OPG expression determined by RT-PCR by using primers specific for OPG and β-actin. (*E*) MM subverts OPG function *in vitro*. TRANCE (1 μg/ml) triggers the development of OCs from precursors present in CSF-1-treated murine marrow (column 2). OPG (1 μg/ml) inhibits TRANCE-induced osteoclastogenesis (column 3). Human MM cell lines ARH-77, U266, H929, RPMI 8226, and ARP-1 partially overcome the suppressive effect of OPG (columns 4–8). Osteoclastogenesis is assessed semiquantitatively by using a colorimetric assay for TRAP with results expressed as $OD_{405\ nm}$. (*F*) MM-induced osteoclastogenesis is TRANCE dependent. Coculture of primary stroma with MM (ARP-1) triggers the generation of OCs from CSF-1-treated murine marrow (BM) (column 2). OCs fail to develop in the absence of MM (column 1), in the presence of 1 μg/ml RANK-Fc (column 3), or if TRANCE-deficient mice (TRANCE −/−, column 4), rather than wild-type littermates, are used as the source of stromal cells. OCs do not develop in the absence of marrow or stroma (not shown).

sion in association with decreased OPG expression is not found in marrow specimens from normal individuals or patients with non-MM B cell malignancies (Fig. 1*B*). Specimens from three patients with MGUS demonstrated intermediate changes in TRANCE and OPG expression.

Deregulation of TRANCE and OPG by MM *in Vitro*. MM induces stromal TRANCE expression *in vitro*. Primary murine stromal cells express TRANCE when cocultured with human MM cell lines (Fig. 2*A*) or when cultured in the presence of media conditioned by stroma–MM coculture (Fig. 2*B*), but not when cultured with media conditioned by MM alone (data not shown). These results suggest that both direct MM–stroma cell contact and soluble factors contribute to TRANCE induction by MM. The suppression of OPG observed in marrow from MM patients also seems to be a direct effect of MM. In coculture, MM cell lines inhibit constitutive OPG mRNA expression by the human osteosarcoma cell line MG63 (Fig. 2*C*). In addition, the induction by TGF-β1 of OPG mRNA expression in the human osteosarcoma cell line U2OS (Fig. 2*D*) is prevented by coculture with MM cell lines. MM can also subvert the function of OPG as a TRANCE antagonist (Fig. 2*E*). Addition of TRANCE to CSF-1-treated murine marrow triggers the development of OCs (24). Although OPG inhibits TRANCE-induced osteoclastogenesis, this inhibition is partially overcome by coculture with human MM cell lines.

MM-Triggered Osteoclastogenesis Depends on TRANCE. The functional consequence of MM-induced TRANCE expression is

demonstrated by the ability of MM–stroma cell cocultures to trigger the *in vitro* generation of OCs from murine marrow. Coculture of murine marrow with primary murine stromal cells and the human MM cell lines H929, RPMI 8226, or ARP-1 results in the development of OCs, evident as multinucleated giant cells expressing TRAP. OCs do not develop if RANK-Fc, a synthetic TRANCE antagonist, is added to the culture, or if TRANCE-deficient mice are used as the source of stromal cells. Results of ARP-1-triggered osteoclastogenesis are presented (Fig. 2*F*).

MM-Induced Bone Destruction Requires TRANCE. In mice with severe combined immunodeficiency (SCID) injected intravenously with the human MM cell line ARH-77, osteolytic lesions develop in 100%, and hind limb paralysis develops in 80% (18, 19). We injected SCID/ARH-77 mice with either the synthetic TRANCE antagonist, RANK-Fc, or $hIgG_1(\lambda)$ (200 μg three times weekly) starting the day after injection of ARH-77. At 6 weeks, RANK-Fc-treated mice exhibited significantly less bone turnover, as measured by urinary Dpd excretion ($P < 0.01$; Fig. 3*A*). Animals treated with $hIgG_1$ also developed obvious radiographic evidence of skeletal destruction, whereas RANK-Fc-treated animals did not (Fig. 3*B*). Over a 7-week period, four of the five mice that received $hIgG_1$ became unable to move their hind limbs, whereas none of the four mice treated with RANK-Fc developed paralysis ($P < 0.01$; Fig. 3*C*). At autopsy, many of the vertebral bodies of the $hIgG_1$-treated mice were infiltrated by MM, with evidence of bony destruction and enlargement because of tumor growth. In contrast, vertebral bodies from RANK-Fc-treated animals were grossly intact. Despite this difference in bone destruction, both groups had comparable titers of $hIgG_1(\kappa)$, the antibody produced by the ARH-77 cell line (Fig. 4*A*). ARH-77 is an Epstein–Barr virus-transformed cell line derived from a patient with plasma cell leukemia (26). Unlike primary MM, growth of ARH-77 in SCID mice is not limited to bone; extraosseous growth of ARH-77 cells was similar in both RANK-Fc and $hIgG_1$-treated animals.

In a second model of MM-associated bone disease, we used SCID-hu mice injected with marrow from three MM patients. In this SCID-hu–MM model, the human bone xenograft becomes infiltrated by MM and eroded by OCs, demonstrating pathology similar to that seen in MM patients (20, 21). Three pairs of SCID-hu mice were generated, with each pair receiving aliquots of marrow from a single individual. Injection of either RANK-Fc or $hIgG_1$ (200 μg three times weekly) was started when titers of MM paraprotein were detected in both mice of a pair. In each case, the mouse with the higher paraprotein titer received RANK-Fc. After 8 weeks, resorption of the xenograft was evident radiographically in all three mice treated with $hIgG_1$ but in none of the mice treated with RANK-Fc (Fig. 3*D*). Consistent with this result, xenografts from mice treated with RANK-Fc contained significantly fewer OCs than did xenografts taken from mice that received IgG_1 ($P < 0.001$; Fig. 3*E*).

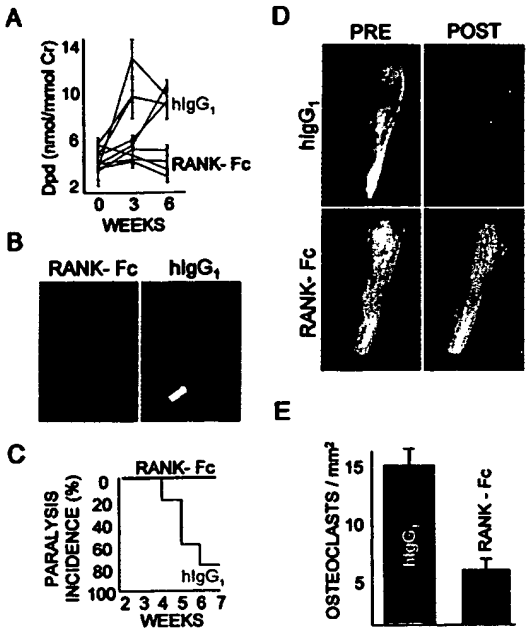

Fig. 3. TRANCE inhibition by RANK-Fc blocks MM-induced bone destruction in two murine models. Irradiated (200 cGy) SCID mice were injected intravenously with 10^6 cells of the human MM line ARH-77. Intravenous administration of either RANK-Fc or $hIgG_1(\lambda)$, both at 200 μg three times weekly, began the following day. At 3 and 6 weeks, both groups had comparable titers of $hIgG_1(\kappa)$, the antibody produced by the ARH-77 cell line. (*A*) Urinary excretion of crosslinked Dpd in SCID/ARH-77 mice. Bone turnover was assessed at 0, 3, and 6 weeks by measuring urinary excretion of Dpd. At 6 weeks, mice treated with RANK-Fc exhibited significantly less bone turnover ($P < 0.01$, Student's *t* test). (*B*) Osteolysis in SCID/ARH-77 mice. Osteolysis was evident in $hIgG_1$ but not RANK-Fc-treated animals. Representative radiographs taken following 6 weeks of therapy are shown. (*C*) Incidence of hind limb paralysis in SCID/ARH-77 mice. Over 7 weeks, four of the five mice that received $hIgG_1$ developed hind limb paralysis as a result of vertebral bone destruction, whereas none of the mice treated with RANK-Fc developed paralysis ($P < 0.01$, Student's *t* test). (*D*) Xenograft osteolysis in SCID-hu–MM mice. SCID-hu mice inoculated with primary MM were treated with either RANK-Fc or $hIgG_1$ (200 μg three times weekly). Injections began when titers of MM paraprotein were detected in both mice of a pair, the mouse with the higher titer receiving RANK-Fc. Osteolysis of the xenograft was evident in $hIgG_1$ but not RANK-Fc-treated animals. Radiographs taken before (PRE) and following (POST) 8 weeks of therapy are shown for one pair of mice. (*E*) Osteoclastogenesis in the xenografts of SCID-hu–MM mice. Xenografts from SCID-hu–MM mice were removed after treatment with either $hIgG_1$ or RANK-Fc and stained for TRAP. Xenografts taken from RANK-Fc recipients had significantly fewer TRAP+ multinucleated giant cells (OCs) per mm^2 than did xenografts taken from $hIgG_1$ recipients ($P < 0.001$, Student's *t* test).

MM Tumor Progression Depends on TRANCE. RANK-Fc treatment caused a marked reduction in serum paraprotein in the SCID-hu–MM mice (Fig. 4*A*), accompanied by a reduction in tumor burden assessed histologically. These findings were not observed in RANK-Fc-treated SCID/ARH-77 mice, consistent with the observed dependence of primary MM, but not plasma cell leukemia, on bone and marrow microenvironments for growth and survival. RANK-Fc does not seem to decrease MM tumor burden through a direct effect of TRANCE or RANK-Fc on plasma cell survival. *In vitro*, addition of TRANCE or RANK-Fc to cultures of primary MM or MM cell lines has no effect on cell growth or survival after exposure to cytotoxic agents (data not shown). The reduced tumor burden in RANK-Fc-treated SCID-hu–MM mice was accompanied by restoration of OPG and reduction of TRANCE expression within the xenograft, further supporting the causal association of MM with altered expression of TRANCE and OPG (Fig. 4*B*).

Discussion

The interaction of MM with marrow stroma is thought to influence both osteoclastogenesis and survival of the malignant clone (1–5). We present evidence that the interaction of MM with stroma results in deregulation of the TRANCE–OPG cytokine axis and that this deregulation is necessary for both MM-associated bone destruction and tumor progression.

Severe bone destruction is a prominent feature of MM but is infrequently associated with other hematologic malignancies. Consistent with this observation, we find marked alteration in

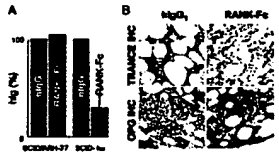

Fig. 4. Effect of RANK-Fc on primary MM in SCID-hu mice. (*A*) Reduction in MM paraprotein. SCID-hu–MM mice demonstrate significantly less MM paraprotein when treated with RANK-Fc ($P < 0.01$, Student's t test), whereas paraprotein levels in SCID/ARH-77 mice are not affected by RANK-Fc treatment. For comparison among animals inoculated with MM from different sources, paraprotein levels for RANK-Fc-treated animals are presented as the percentage (mean ± SE) of the paraprotein level measured in the $hIgG_1$-treated animals. (*B*) OPG and TRANCE expression in xenografts from SCID-hu–MM mice (IHC, ×600). Immunohistochemistry performed on xenografts harvested from SCID-hu–MM mice after 8 weeks of treatment demonstrates normalization of TRANCE and OPG expression in the xenografts taken from RANK-Fc-treated animals.

expression of both TRANCE and OPG only in specimens from MM patients. In marrow infiltrated by MM, TRANCE is expressed by stromal and activated T cells at the interface of MM with normal marrow elements. The absence of TRANCE staining in light chain-expressing cells in specimens of plasmacytoma and MM-replaced bone marrow suggests that malignant plasma cells do not themselves produce TRANCE, a conclusion supported by our inability to document TRANCE mRNA or protein in MM cell lines. Our coculture experiments also indicate a stromal, rather than plasma cell, origin for TRANCE; MM can induce development of OCs from marrow precursors in the presence of wild-type, but not TRANCE-deficient, stroma.

MM must interact directly with stroma to initiate deregulation of TRANCE expression. *In situ*, TRANCE is expressed at the interface of MM with normal marrow elements. *In vitro*, TRANCE expression by stroma is induced by coculture with MM but not by addition of MM-conditioned media. This requirement for direct cellular contact is consistent with the study of Michigami *et al.*, who report enhanced OC-stimulating activity by the ST2 stromal cell line after direct contact with 5TGM1 murine myeloma cells (27). *In vitro*, TRANCE expression can also be induced by media conditioned by MM–stroma coculture. Thus, MM–stroma contact may trigger the production of TRANCE-inducing cytokines. Several MM-associated OC-activating factors (OAFs) including IL-1β, produced by malignant plasma cells following interaction with marrow stroma, and IL-6 and IL-11, produced by stroma in response to MM, have been shown *in vitro* to induce stromal expression of TRANCE (28). Other OAFs, such as IL-17 and hepatocyte growth factor, induce stromal expression of IL-11 and may act indirectly to increase TRANCE (29).

The Significance of TRANCE Deregulation by MM Can Be Demonstrated Both *In Vitro* and *In Vivo*. In coculture, OC development in response to MM requires TRANCE expression by stromal cells and is blocked by addition of the TRANCE antagonist RANK-Fc. *In vivo*, administration of RANK-Fc abrogates the development of bony disease in both SCID mice inoculated with the IL-1β-producing ARH-77 cell line and in SCID-hu mice inoculated with primary MM. Taken together, these results indicate that TRANCE is essential for MM-triggered osteoclastogenesis and that antagonism of TRANCE activity can block MM-associated bone destruction.

The ability of RANK-Fc to block osteoclastogenesis both *in vitro* and *in vivo* emphasizes the importance of the concurrent decrease in OPG expression we observe in MM marrow specimens. OPG, the natural TRANCE decoy receptor, is central to the maintenance of bone homeostasis (9, 11) and can inhibit osteoclastogenesis and bone loss after administration of osteotrophic agents (30–32). The high level of OPG expression we observe in normal marrow would be expected to counteract moderate increases in TRANCE. Therefore, the ability of MM to concurrently down-regulate expression of this natural buffer may be critical to the development of bone disease. *In vitro*, we find that constitutive stromal expression of OPG is reduced by coculture with MM, which may reflect the ability of OAFs, including IL-1β, IL-6, and IL-11, to inhibit transcription of OPG (28). We also find that MM interferes with up-regulation of OPG by TGF-β, which may reflect the ability of OAFs, including hepatocyte growth factor, IL-1β, and TNF-α, to inhibit TGF-β signaling through activation of either mitogen-activated protein kinase or the inhibitory Smad7 (33). TGF-β, which is released from bone matrix during OC resorption, is thought to act as a feedback regulator to control osteoclastogenesis by stimulating stromal expression of OPG and inhibiting stromal expression of TRANCE (34–36). However, TGF-β can also stimulate the growth and maturation of OCs already primed by TRANCE (37). Our finding that MM directly inhibits TGF-β-induced expression of OPG is of particular importance in light of reports that MM cells can produce TGF-β (38) and suggests that MM might simultaneously use TGF-β to enhance osteoclastogenesis, yet block modulation by TGF-β of stromal TRANCE and OPG expression. Our coculture experiments also suggest subversion of OPG function by MM because addition of MM cell lines partially overcomes TRANCE inhibition by OPG. The mechanisms responsible for this activity are unknown, but they may involve the ability of syndecan-1, expressed at high level on the surface of malignant and nonmalignant plasma cells, to bind the heparin-binding domain of OPG (39). It is of particular interest that although all of the MM cell lines we tested increase TRANCE expression, not all interfere with OPG expression by stroma, suggesting that some factors controlling OPG expression are independent of controls on TRANCE.

Our data suggest that the ability to disrupt the TRANCE–OPG cytokine axis, an ability shared by many MM-associated OAFs, is critical to the development of MM-associated bone destruction. Other OAFs, notably VEGF, which activates the CSF-1 receptor c-fms (40), a necessary step in OC development, MIP-1α, which recruits OC precursors (41), and TNF-α, which augments the intracellular signals initiated by TRANCE–RANK interaction (42), seem to act in synergy with TRANCE to stimulate osteoclastogenesis.

Disruption of the TRANCE–OPG axis by MM may also be necessary for tumor progression. Administration of RANK-Fc results in decreased tumor burden in the SCID-hu model of human MM, suggesting a role for TRANCE in MM cell growth. However, unlike other stromally expressed growth factors induced by hematologic malignancies, such as granulocyte colony-stimulating factor, induced by myeloid leukemias, or IL-6, induced by B cell malignancies, TRANCE does not act as a direct survival factor for plasma cells. Rather, TRANCE seems to act indirectly to support MM as part of a paracrine loop involving stroma, OCs, and bone. Inhibition of TRANCE-induced osteoclastogenesis may decrease OC production of cytokines such as IL-6 that are known MM survival factors, it may limit the release during bone resorption of MM growth factors such as IGF-1 (43), or it may simply limit the niche for MM growth. Similar mechanisms have been described for bisphosphonates (44). A reciprocal relationship between osteoclastogenesis and early B lymphopoiesis has been suggested (45–47); our data indicate that osteoclastogenesis and survival of MM cells are also interdependent.

In conclusion, we find that MM deregulates stromal TRANCE and OPG expression to trigger bone destruction and promote tumor growth. The multiple mechanisms used by MM to control

expression of TRANCE and OPG underscore the critical role of this axis in MM biology.

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Osteoprotegerin inhibits prostate cancer–induced osteoclastogenesis and prevents prostate tumor growth in the bone

See related Commentary on pages 1219–1220.

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Prostate cancer (CaP) forms osteoblastic skeletal metastases with an underlying osteoclastic component. However, the importance of osteoclastogenesis in the development of CaP skeletal lesions is unknown. In the present study, we demonstrate that CaP cells directly induce osteoclastogenesis from osteoclast precursors in the absence of underlying stroma in vitro. CaP cells produced a soluble form of receptor activator of NF-κB ligand (RANKL), which accounted for the CaP-mediated osteoclastogenesis. To evaluate for the importance of osteoclastogenesis on CaP tumor development in vivo, CaP cells were injected both intratibially and subcutaneously in the same mice, followed by administration of the decoy receptor for RANKL, osteoprotegerin (OPG). OPG completely prevented the establishment of mixed osteolytic/osteoblastic tibial tumors, as were observed in vehicle-treated animals, but it had no effect on subcutaneous tumor growth. Consistent with the role of osteoclasts in tumor development, osteoclast numbers were elevated at the bone/tumor interface in the vehicle-treated mice compared with the normal values in the OPG-treated mice. Furthermore, OPG had no effect on CaP cell viability, proliferation, or basal apoptotic rate in vitro. These results emphasize the important role that osteoclast activity plays in the establishment of CaP skeletal metastases, including those with an osteoblastic component.

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Introduction

Prostate cancer is the most frequently diagnosed cancer in men and the second leading cause of cancer death among men in the US. The most common site of prostate cancer metastasis is the bone, with up to 84% of patients demonstrating skeletal metastases (1). While initially thought to be primarily osteoblastic, it is now recognized that prostate cancer skeletal metastases have an extensive bone resorptive component (2, 3) that is caused primarily by osteoclasts (4). This accounts, in part, for the ability of bisphosphonates, which are anti-osteoclastogenic agents, to diminish osteolysis, decrease pain, and improve mobility in patients with prostate cancer skeletal metastasis (5). However, the mechanisms through which prostate cancer skeletal metastases induce osteolytic lesions are not defined.

The presence of an osteolytic component in prostate cancer skeletal metastases suggests that osteoclastogenesis may play a role in the establishment of these lesions. Recently, the discovery and characterization of a novel cytokine system — the TNF family member, receptor activator of NF-κB ligand (RANKL, also called OPGL, TRANCE, and ODF); its receptor, receptor activator of NF-κB (RANK, also called ODAR); and its decoy receptor, osteoprotegerin (OPG, also called OCIF and TR1) — has established a common mechanism through which osteoclastogenesis is regulated in normal bone (reviewed in ref. 6). RANKL, a transmembrane molecule located on bone marrow stromal cells and osteoblasts, binds to RANK, which is located on the surface of osteoclast precursors. This ligand-receptor interaction activates NF-κB, which stimulates differentiation of osteoclast precursors to osteoclasts. OPG, also produced by osteoblasts/stromal cells, binds to RANKL, sequestering it from binding to RANK, which results in inhibition of osteoclastogenesis. The requirement for RANKL to induce osteoclastogenesis suggests that it may mediate the osteolytic component of prostate cancer skeletal lesions. However, it is currently unknown if prostate cancer uses the

RANKL:RANK axis to induce osteolysis. Furthermore, OPG has been shown to inhibit primary bone sarcoma–induced osteolysis and tumor-induced bone pain, but not tumor burden in mice (7). However, the role of OPG on tumors metastatic to bone or epithelial tumors remains undetermined. Accordingly, in the current study, we investigated the mechanism through which prostate cancer induces osteoclastogenesis and determined if OPG could inhibit establishment of prostate tumor in murine bone.

Methods

Animals. Eight-week-old male SCID and C57BL/6 mice (Charles River, Wilmington, Massachusetts, USA) were housed under pathogen-free conditions in accordance with the NIH guidelines using an animal protocol approved by the University of Michigan Animal Care and Use Committee.

Cell lines. The human prostate cell line LNCaP (American Type Tissue Collection, Manassas, Virginia, USA) derived from an aspirate of a subcutaneous supraclavicular lymph node prostate cancer metastases, was maintained in RPMI-1640 supplemented with 10% FBS, 100 U/l penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine. LNCaP cells induce very low levels of blastic activity when implanted into bone and do not readily metastasize (8). C4-2B cells (UroCor Inc., Oklahoma City, Oklahoma, USA) are derived from LNCaP cells after several passages through nude mice and aggressive tumors that metastasize to bone (8, 9). The C4-2B cells were maintained in T medium, which consisted of 80% DMEM (Life Technologies Inc., Grand Island, New York, USA), 20% F12K (Irving Scientific, Santa Ana, California, USA), 3 g/l $NaHCO_3$, 100 U/l penicillin G, 100 μg/ml streptomycin, 5 μg/ml insulin, 13.6 pg/ml triiodothyronine, 5 μg/ml apo-transferrin, 0.25 μg/ml biotin, 25 μg/ml adenine, and were supplemented with 10% FBS. The human osteogenic sarcoma cell line SaOS (American Type Tissue Collection), was maintained in DMEM supplemented with 10% FBS, 100 U/l penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine. Murine monocyte/macrophage-like cell line, RAW 264.7, commonly used as an osteoclast precursor cell line (American Type Tissue Collection), was maintained in RPMI-1640 supplemented with 10% FBS, 100 U/l penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine.

Tumor implant. Single-cell suspensions (3×10^5 cells) of C4-2B cells in T media were injected into the right tibia of 8-week-old male SCID mice (n = 30) as described previously (10). Briefly, mice were anesthetized (135 mg ketamine, 15 mg xylazine/kg body weight), the knee was flexed, and a 26-g, 3/8-inch needle was inserted into the proximal end of right tibia followed by injection of 20 μl of the cell suspension.

Subcutaneous tumors. At the same time as intratibial injection, C4-2B cells were resuspended in T media plus 10% FBS. Two million cells were mixed 1:1 with Matrigel (Collaborative Biomedical Products, Bedford, Massachusetts, USA), and then injected in the flank at 100 μl/site using a 23-g needle. Subcutaneous tumor growth was monitored by palpation, and two perpendicular axes were measured; the tumor volume was calculated using the formula as described previously (11): volume = length × width^2/2.

Treatment. At the time of injection, mice were randomized to receive either injections (through tail vein) of vehicle (1% BSA in 1× PBS) (n = 10) or recombinant mouse OPG/Fc chimera (R&D Systems Inc., Minneapolis, Minnesota, USA) at 2 mg/kg body weight (n = 10) twice a week and continued for 4 weeks. Tumors were allowed to grow for 16 weeks, and at the end of week 16, all animals were sacrificed. One animal in the OPG treatment group died 1 day after the tumor was implanted. To evaluate histology at a 4-week time point, we performed the same protocol with another five vehicle-treated and five OPG-treated mice. Before sacrifice, the animals were anaesthetized, and magnified flat radiographs were taken with a Faxitron (Faxitron X-Ray Corp., Wheeling, Illinois, USA). At sacrifice, all of the major organs and lumbar vertebrae were harvested for histological analysis.

Histopathology and bone histomorphometry. Histopathology was performed as we have described previously (12). Briefly, bone specimens were fixed in 10% formalin for 24 hours, then decalcified using 12% EDTA for 72 hours. The specimens were then paraffin embedded, sectioned (5 μM), and stained with hematoxylin and eosin to assess histology or stained with tartrate-resistant acid phosphatase (TRAP) to identify osteoclasts. To perform TRAP staining, nonstained sections were deparaffinized and rehydrated, then stained for TRAP (Acid Phosphatase Kit 387-A; Sigma Diagnostics, St. Louis, Missouri, USA) as directed by the manufacturer, with minor modification. Briefly, the specimens were fixed for 30 seconds and then stained with acid phosphatase and tartrate solution for 1 hour at 37°C, followed by counterstaining with hematoxylin solution. Osteoclasts were determined as TRAP-positive staining multinuclear (>3 nuclei) cells using light microscopy. Histomorphometric analysis was performed on a BIOQUANT system (BIOQUANT-R&M Biometrics Inc., Nashville, Tennessee, USA). The osteoclast perimeter (osteoclast number per millimeter of bone) in vehicle-treated animals compared with normal bone surface in the OPG-treated animals was quantified, without knowledge of treatment group, by examination at ×200. For routine histopathology, soft tissues were preserved in 10% formalin, embedded in paraffin, sectioned (5 μM), and stained with hematoxylin and eosin.

Prostate-specific antigen immunohistochemistry. Nonstained sections were deparaffinized and rehydrated then stained for prostate-specific antigen (PSA) with anti-human PSA Ab using standard immunohistochemistry techniques. Human prostate cancer tissue and normal prostate tissue were used as a positive control and a negative control, respectively.

Obtaining conditioned media. Conditioned media (CM) was obtained from LNCaP or C4-2B cells by plating 5×10^6 cells in 10-cm tissue culture dishes for 12 hours in T media with 10% FBS. The media was then changed to 10 ml of RPMI plus 0.5% FBS, and supernatants were collected 24 hours later. To normalize for differences in cell density due to proliferation during the culture period, cells from each plate were collected, and total DNA content/plate was determined (spectrophotometric absorbance at 260 nm). CM was then normalized for DNA content between samples by adding RPMI.

Assessment of ability of prostate cancer cells to induce osteoclastogenesis in the presence of osteoblast/stromal cells in vitro. To establish cocultures, single cell suspensions (10^5 cells/well) of LNCaP or C4-2B cells were plated on sterile glass coverslips in 24-well plates in RPMI or T media plus 10% FBS. Cells were grown for 12 hours, then media was changed to RPMI plus 0.5% FBS. Then, all wells were overlaid with single-cell suspension of murine bone marrow cells (10^6 cells in 1 ml media) from six healthy C57BL mice. In addition to the cocultures, CM at different concentrations was added directly to murine bone marrow cells in the absence of prostate cancer cells. Vitamin D was not added to either the cocultures or the CM cultures. Recombinant OPG (R&D Systems Inc.) in indicated concentrations, vehicle (1% BSA in 1× PBS), or M-CSF (1 ng/ml) (Sigma Diagnostics) was added to the cultures. The cultures were maintained for 9 days with replacement of half the medium (including the different concentrations of CM with indicated treatments) every 3 days. Samples were evaluated in quadruplicates. Osteoclast-like cells were identified as TRAP-positive multinucleated (>3 nuclei) cells. Results were reported as the number of osteoclast-like cells per coverslip.

Assessment of ability of prostate cancer cells to induce osteoclastogenesis in the absence of osteoblast/stromal cells in vitro. Single-cell suspensions (10^5 cells/well) of RAW 264.7 cells were plated on sterile glass coverslips in 24-well plates in RPMI plus 10% FBS. Cells were grown for 12 hours then the media was changed to RPMI plus 0.5% FBS. CM (25%; CM volume/total culture volume) from C4-2B cells was added to the RAW 264.7 cell cultures. Additionally, recombinant human RANKL (10 ng/ml; PeproTech Inc., Rocky Hill, New Jersey, USA), recombinant OPG (R&D Systems Inc.) as indicated, or vehicle (1% BSA in 1× PBS) was added. These cultures did not contain vitamin D. The cultures were maintained for 7 days with replacement of half the medium (including 25% CM with indicated treatment) at day 3. Samples were evaluated in quadruplicate. Osteoclast-like cells were identified as TRAP-positive multinucleated (>3 nuclei) cells. Results were reported as the number of osteoclast-like cells per coverslip.

RANKL mRNA expression. Total RNA from LNCaP and C4-2B cells was collected (Trizol reagent; Life Technologies Inc.), then subjected to PCR for detection of RANKL mRNA. PCR primers used for detection of RANKL consisted of sense, 5′-GCTTGAAGCTCAGCCTTTTGCTCAT-3′, and antisense, 5′-GGGGTTGGAGACCTCGATGCTGATT-3′, resulting in a PCR product of 412 bp (primer sequence kindly provided by J. Brown, University of Washington, Seattle, Washington, USA). The human osteoblastic-like/osteosarcoma SaOS cell line was used as a positive control for OPGL expression. RT-PCR was performed with 1 μg of total RNA using the Access RT-PCR system (Promega Corp., Madison, Wisconsin, USA), as directed by the manufacturer, in a thermal cycler (GeneAmp PCR system 9700; Perkin-Elmer Applied Biosystems, Foster City, California, USA) under the following conditions: first-strand cDNA was synthesized at 48°C for 45 minutes; then denatured at 94°C for 2 minutes for the first cycle and at 15 seconds for additional 35 cycles; annealing was performed at 55°C for 30 seconds; and extension at 72°C for 60 seconds. Final extension was at 72°C for 5 minutes. The PCR products were subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide.

Western blot analysis. To evaluate for RANKL in the prostate cancer cell supernatant, CM collected from LNCaP and C4-2B cell cultures were concentrated 100-fold using a 10-kDa cut-off Microcon centrifugal filter devices (Amicon Inc., Beverly, Massachusetts, USA). To evaluate for RANKL expression in the prostate cancer cells, confluent LNCaP and C4-2B cells were washed twice with PBS and then lysed in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with 100 ng/ml PMSF. Proteins (50 μg/lane) from the concentrated CM and cell lysates were applied to SDS-PAGE followed by Western blot analysis with rabbit anti-human soluble RANKL polyclonal Ab (PeproTech Inc.). The Ab binding was revealed using an HRP-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) and enhanced chemiluminescence (ECL) blot detection system (Amersham Pharmacia Biotech).

Cell viability. C4-2B cells were plated at 2×10^6/plate in 60-mm plates in triplicate with T media. After 12 hours of culture, media was changed to RPMI plus 0.5% FBS, and recombinant OPG (R&D Systems Inc.) was added at different concentrations (0–100 ng). Subsequently, cells were harvested at 24 hours and viability was examined by trypan blue exclusion.

Cell proliferation. Cell proliferation was measured using the CellTiter 96 AQ nonradioactive cell proliferation assay (Promega Corp.). Briefly, C4-2B cells in T media were added to the wells of a 96-well plate at 5,000/well in triplicates. After 12 hours of culture, the media was changed to RPMI plus 0.5% FBS and a different concentration (0–100 ng) of recombinant OPG (R&D Systems Inc.) was added. Cells were allowed to grow for 24 hours, then 20 μl/well of combined MTS/PMS solution was added. After incubation of 1 hour at 37°C in a humidified 5% CO_2 atmosphere, the absorbance at 490 nm was recorded by using an ELISA plate reader.

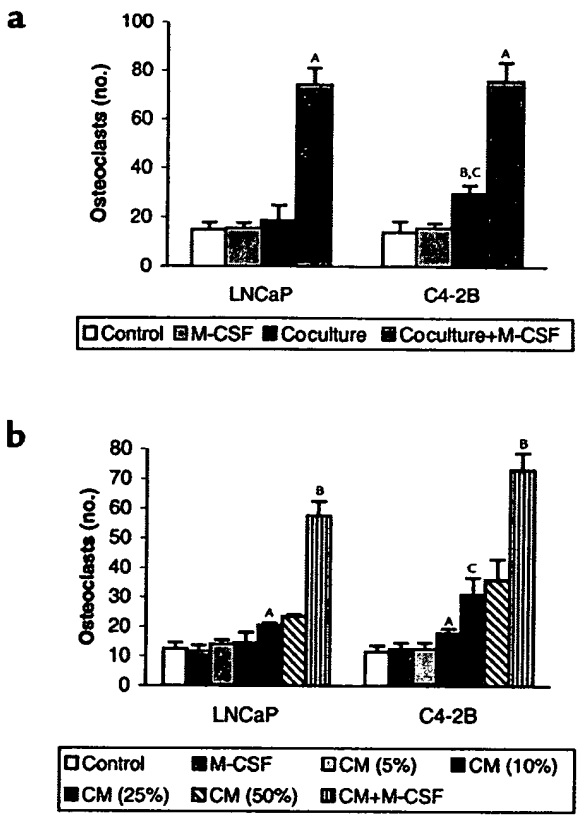

Figure 1
OPG inhibits LNCaP and C4-2B cell-induced osteoclastogenesis of osteoblast/stromal cells in vitro. (**a**) LNCaP or C4-2B cells were directly cocultured with murine bone marrow cells for 9 days in the presence or absence of M-CSF (1 ng/ml). Osteoclast-like cells were identified as TRAP-positive multinucleated (>3 nuclei) cells. $^{A}P < 0.001$ compared with its respective control culture (without adding CaP cells) or coculture; $^{B}P < 0.01$ compared with its respective control culture; $^{C}P < 0.01$ compared with its LNCaP cells. (**b**) Conditioned media (CM) from LNCaP and C4-2B cells was collected after 24 hours of culture, then the indicated concentrations of CM (vol/vol) was added to murine bone marrow cells and cultured for 9 days. Osteoclast-like cells were identified as TRAP-positive multinucleated (>3 nuclei) cells. $^{A}P < 0.001$ compared with respective control culture (without adding CM); $^{B}P < 0.001$ compared with each cell line's respective control culture or coculture; $^{C}P < 0.01$ compared with its LNCaP cells. (**c**) CM (25% vol/vol) from LNCaP and C4-2B cells were collected after 24 hours of culture, then added to murine bone marrow cells with different dose of recombinant mouse OPG (1–1000 ng/ml) as indicated and cultured for 9 days. Osteoclast-like cells were identified as TRAP-positive multinucleated (>3 nuclei) cells. $^{A}P < 0.001$ compared with its control culture; $^{B}P < 0.01$ compared with its respective vehicle-treated CM cultures; $^{C}P < 0.001$ compared with its respective vehicle-treated CM cultures. All in vitro cultures were evaluated in quadruplicate. Results were reported as the mean (± SD) number of osteoclast-like cells per coverslip. Data were analyzed using ANOVA and Fisher's least-significant difference for post hoc analysis.

Cell apoptosis. C4-2B cells were plated at 10^6/well in 12-well plates in triplicate with T media. After 12 hours of culture, media was changed to RPMI plus 0.5% FBS and immediately a different concentration (0–100ng) of recombinant OPG (R&D Systems Inc.) was added. Subsequently, cells were harvested at 24 hours, and apoptosis was assessed by flow cytometry using Annexin V-FITC detection Kit (PharMingen, San Diego, California, USA) following the manufacturer's protocol.

Data analysis. Fisher's exact test was used to determine if there was a difference in the incidence of tumor development between groups. ANOVA was used for the in vitro culture system to evaluate differences in prostate cancer cell-induced osteoclastogenesis. Fisher's least-significant difference was used for post hoc analysis. Student's *t* test was used for bivariate comparisons. P values less than or equal to 0.05 were considered to be statistically significant.

Results

The mechanism through which prostate cancer induces osteolysis at its skeletal metastatic site has not been defined. To test whether prostate cancer cells induce osteoclastogenesis in vitro, LNCaP and C4-2B cells were directly cocultured with murine bone marrow cells for 9 days in 1,25 $(OH)_2$ vitamin D_3-free (VitD-free) media. LNCaP and C4-2B cells induced approximately 30% and 90% increase of osteoclasts compared with marrow control cultures without prostate cancer cells, respectively (Figure 1a). The addition of M-CSF, a strong costimulator of osteoclastogenesis, synergistically enhanced the prostate cancer cell-induced osteoclastogenesis (Figure 1a). A variety of soluble factors such as IL-6, PTHrP, and soluble RANKL may act to induce osteoclastogenesis (13). Thus, to determine if the prostate cancer cells induced osteoclastogenesis through a soluble factor, we tested the ability of CM from LNCaP and C4-2B cell cultures to induce osteoclastogenesis in vitro. CM was added to murine bone marrow cells and culture was maintained for 9 days. The CM induced osteoclastogenesis in a dose-responsive fashion (Figure 1b). C4-2B cells induced approximately 80% more osteoclastogenesis than its parental LNCaP cells. These results demonstrated that the prostate cancer cell lines produce soluble factors that induce osteoclastogenesis. Because RANKL is a key osteoclastogenic factor that has been reported to exist in a soluble form (14–18), we next assessed if the prostate cancer cell lines' pro-osteoclastogenic activ-

ity could be blocked by OPG, a RANKL decoy receptor. The addition of OPG diminished the prostate cancer cell CM-induced osteoclastogenesis in a dose-dependent manner for both cell lines (Figure 1c). The ability of OPG to inhibit prostate cancer cell CM-induced osteoclastogenesis suggested that the prostate cancer cells induced osteoclastogenesis through sRANKL. However, these studies did not differentiate if the RANKL activity was derived directly from the prostate cancer cells or if the prostate cancer cells produced a soluble factor that induced RANKL from cells in the bone marrow stroma present in the murine bone marrow culture system. To differentiate between these possibilities, we tested the ability of C4-2B cell CM to induce osteoclastogenesis in a macrophage-like osteoclast precursor cell line, RAW 264.7, in the absence of supporting osteoblast/stromal cells. We found that exogenous (human recombinant) sRANKL itself can stimulate osteoclast formation in this in vitro culture system. Furthermore, CM from the C4-2B cells induced osteoclast formation that was inhibited by OPG in a dose-dependent manner. (Figure 2, a and b). These data provided strong evidence that the prostate cancer cells themselves produce active sRANKL. To confirm that possibility, we determined if the LNCaP and C4-2B cells expressed RANKL mRNA and protein. We found that both cell lines expressed RANKL mRNA (Figure 3a) and full-length RANKL protein (Figure 3b), as did the SaOS-positive control cell line. Finally, we detected sRANKL in concentrated CM from both LNCaP and C4-2B cells, but not SaOS cells (Figure 3b) at the molecular weight of 26 kDa as previously reported (14, 15). Taken together, these data provide evidence that prostate cancer cells are able to induce osteoclastogenesis directly through production of soluble RANKL.

The observation that OPG blocked C4-2B–induced osteoclastogenesis in vitro provided the rationale to test if OPG could prevent establishment of prostate cancer in bone in vivo. Accordingly, we evaluated the effect of OPG on the growth of C4-2B cells injected intratibially into SCID mice. Additionally, to determine if the effect was specific to bone, the same mice were inoculated with C4-2B cells subcutaneously at the time of intratibial tumor injection. Immediately after tumor cell injection, OPG (2 mg/kg) or vehicle was administered twice a week for 4 weeks through tail-vein injection. There were 15 mice per treatment group. One animal in the OPG treatment group died of unknown causes 1 day after the initial tumor injection. Four weeks after tumor injection, five mice from each group were sacrificed for evaluation. Skeletal lesions could not be identified by radiographs in either the vehicle or OPG-treated group (Table 1 and Figure 4); however, histological analysis revealed PSA-positive tumor infiltration in all the vehicle-treated animals, but not in any of the OPG-treated animals (Table 1 and Figure 4). Furthermore, there was an approximately eightfold increase of osteoclasts at the bone/tumor interface compared with the normal bone osteoclast perimeter observed in OPG-treated mice (Table 2). The osteoclast perimeter in the OPG-treated mice was similar to that in our previous report of osteoclast perimeter in untreated mice (12). The remaining mice were maintained for an additional 12 weeks at which time they

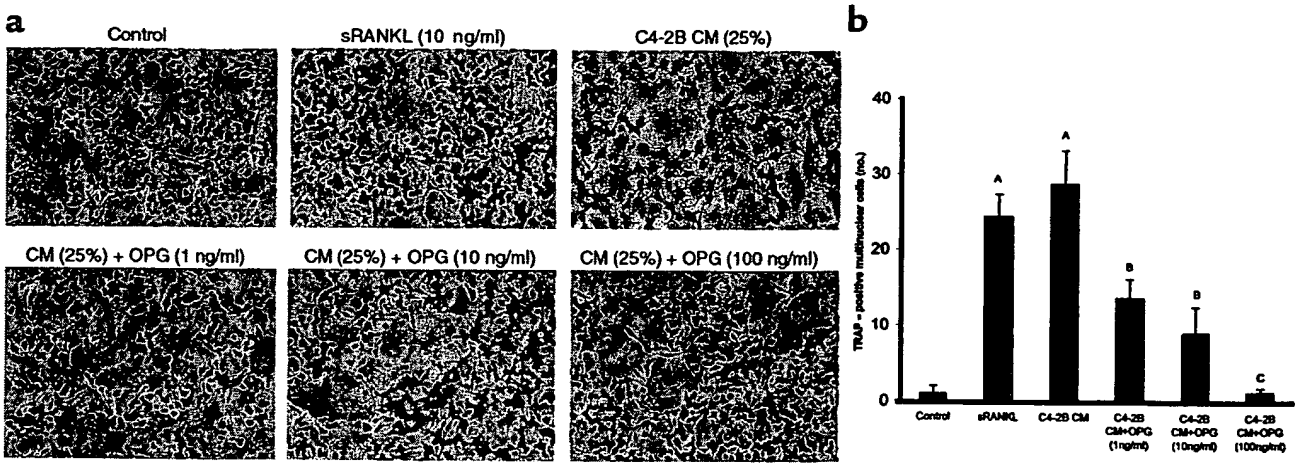

Figure 2

C4-2B CM induces osteoclastogenesis in the absence of osteoblast/stromal cells, and OPG inhibits the osteoclastogenesis in vitro. Single-cell suspensions (10^5 cells/well) of RAW 264.7 cells were plated in a 24-well plate on top of a sterile coverslip in RPMI plus 10% FBS. Cells were grown for 12 hours, then the media was changed to RPMI plus 0.5% FBS. CM from C4-2B cells was harvested (as described in Methods) and added to a final concentration of 25% (vol/vol). Immediately, recombinant human soluble RANKL (10 ng/ml) or the indicated concentration of recombinant mouse OPG or vehicle (1% BSA in PBS) was added. Osteoclasts were identified as TRAP-positive multinucleated (>3 nuclei) cells. (**a**) Representative pictures of cultures stained for TRAP. (**b**) Osteoclasts per coverslip were quantified. Samples were evaluated in quadruplicate. Results are reported as mean (±SD). Data were analyzed using one-way ANOVA. $^{A}P < 0.001$ compared with control culture; $^{B}P < 0.01$ compared with the CM-treated group; $^{C}P < 0.001$ compared with the CM-treated group.

Table 1
OPG prevents establishment of prostate cancer in the skeleton in mice

		4 weeks		16 weeks	
		Vehicle	OPG	Vehicle	OPG
Tibia	Radiologic	0/5	0/5	5/10	0/9[B]
	Histologic	5/5	0/5[A]	7/10	0/9[C]
Subcutis		4/5	5/5	10/10	8/9

Mice were injected intratibially and subcutaneously with C4-2B tumors, and then either vehicle or OPG (2 mg/kg) was administered intravenously twice a week for 4 weeks. The mice were sacrificed at the end of 4 weeks and 16 weeks after the initial injection of tumor. Tibial tumors were evaluated using radiography and histology. Subcutaneous tumors were evaluated by histology. The results are reported as the number of tumors for each group and total number of animals in each group. One mouse in the OPG-treatment died of unknown cause 1 day after the tumor injection, thus there were only nine animals in that group. [A]$P = 0.001$ compared with vehicle-treated animals; [B]$P = 0.03$ compared with vehicle-treated animals; [C]$P = 0.003$ compared with vehicle-treated animals. Data were analyzed by Fisher's exact test.

were sacrificed, and tumor burden was evaluated using radiography and histology. Tumor was not identified in lung, liver, spleen, brain, or vertebrae by histological evaluation. However, radiographs revealed marked osteolytic lesions with occasional foci of strongly osteoblastic lesions in the vehicle-treated animals compared with normal radiographs in the OPG-treated animals (Figure 4). Histology revealed that PSA-positive prostate cancer cells replaced the marrow in the vehicle-treated mice and a heterogeneous mixture of mostly shortened trabeculae with occasional areas consisting of thickened trabeculae (Figure 4). These data confirmed that the PSA-positive C4-2B tumor cells were growing in the vehicle-treated mice. Furthermore, the absence of PSA-positive cells in the OPG-treated mice supports that there was either no or a minimal tumor burden in these mice. Finally, we observed a high number of TRAP-positive osteoclasts at the bone/tumor interface (Figure 5), indicating that mature osteoclasts were directly adjacent to tumor with increased activity. Histomorphometric quantification of osteoclast number at the bone/tumor interface revealed an approximately 15-fold increase of osteoclasts in the vehicle-treated animals compared with normal bone surface in the OPG-treated animals (Table 2). Taken together, these data demonstrate that OPG inhibits the development of C4-2B–derived tumors, including both osteolytic and osteoblastic components. Furthermore, the data strongly suggest that the development of osteoblastic lesions is dependent on osteoclastic activity.

The incidence of tumor growth in the bone at 16 weeks after tumor injection in the vehicle-treated mice was 50% and 70%, based on radiography and histology, respectively (Table 1). In contrast, intratibial tumor was not detected by radiography or histology in the OPG-treated mice (Table 1). Furthermore, in contrast to tumor growth at the bone site, there was no difference in subcutaneous tumor incidence between vehicle-treated and OPG-treated mice (Table 1), and the growth rate of the subcutaneous tumors did not differ between the groups (Figure 6). Taken together, these data demonstrate that OPG preferentially inhibits C4-2B tumor growth in bone. The data also suggest that OPG does not have a direct effect on tumor growth, as the subcutaneous tumors grew similarly in the vehicle-treated and OPG-treated groups. This is further supported by the observation that OPG had no effect on proliferation, cell viability, and basal apoptotic rate of C4-2B cells in vitro (data not shown; experimental protocols were described in Methods).

Discussion

In 1958, Roland introduced the theory that every primary or metastatic cancer in bone (including osteoblastic prostate cancers) begins with osteolysis (19). However, while important gains in understanding the role of osteoclastic activity have been made for osteolytic tumors, the importance of osteoclastic activity in the development of prostate cancer skeletal metastatic lesions has received little attention because of their overall osteoblastic radiographic appearance. Yet, despite the radiographic appearance, it is clear from histological evidence that prostate cancer metastases form a heterogeneous mixture of osteolytic and osteoblastic lesions (2, 20–23). In fact, histomorphometric analysis of metastatic lesions reveals that osteoblastic metastases form on trabecular bone at sites of previous osteoclastic resorption, suggesting that bone resorption is required for subsequent osteoblastic bone formation (2). To test this hypothesis, we determined if inhibiting osteoclastogenesis would prevent establishment of a prostate cancer

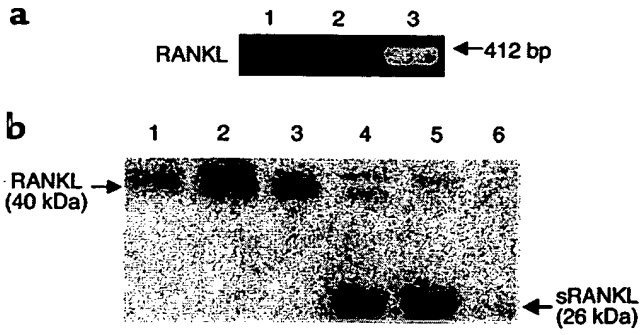

Figure 3
LNCaP and C4-2B cells express RANKL and produce soluble RANKL. (a) One microgram of total RNA from the indicated cells was subjected to RT-PCR. Lanes 1, 2, and 3 are PCR products from LNCaP, C4-2B, and SaOS, respectively. (b) Total cellular protein or CM (concentrated 100-fold using Microcon centrifugal filter devices) from LNCaP, C4-2B, and SaOS cell cultures were subjected to Western blot analysis (50 μg/lane) using rabbit anti-human soluble RANKL polyclonal Ab as primary Ab and HRP-conjugated anti-rabbit IgG as secondary Ab. Bands were detected using luminescence and autoradiography. Lane 1, LNCaP cell lysate; lane 2, C4-2B cell lysate; lane 3, SaOS cell lysate; lane 4, LNCaP concentrated CM; lane 5, C4-2B concentrated CM; and lane 6, SaOS concentrated CM.

Table 2
Histomorphometric quantification of OC perimeter at bone/tumor interface in the vehicle-treated mice compared with normal bone surface in the OPG-treated mice

	4 weeks		16 weeks	
	Vehicle	OPG	Vehicle	OPG
OC perimeter (no. OC/mm)	10.84 ± 2.60	1.40 ± 0.46[A]	20.02 ± 3.68	1.36 ± 0.63[B]

[A]$P < 0.01$ compared with vehicle-treated animals; [B]$P < 0.001$ compared with vehicle-treated animals. Student's t test.

xenograft, which forms mixed osteoblastic and osteoclastic lesions, in the tibia of mice. The results from the present study demonstrate that prostate cancer cells can directly induce osteoclastogenesis through production of sRANKL. Additionally, this study demonstrates that OPG-mediated inhibition of osteoclastogenesis was associated with prevention of C4-2B cell growth in osseous, but not in nonosseous tissue. Finally, the observation that OPG did not diminish subcutaneous growth of the tumor, in combination with the observation that OPG had no direct affect on the prostate cancer cells in vitro suggests that OPG's ability to inhibit prostate cancer establishment was due specifically to factors in the bone microenvironment. These data suggest that inhibition of osteoclast activity is sufficient to diminish the development of skeletal metastatic prostate tumors that have both osteolytic and osteoblastic components.

Our results are consistent with reports that most prostate cancer skeletal metastasis reveals an osteoclastic component (2–4). Based on the data in this report, together with earlier evidence that tumors that metastasize to bone require osteoclastic activity to release tumor-supportive growth factors from bone (reviewed in ref. 24), it appears that osteoclastogenesis is an important mediator of prostate cancer establishment in the bone in this murine model. These results are reflected in clinical data, which demonstrate that systemic markers of bone resorption are increased in men with prostate cancer skeletal metastases (25, 26) and that bisphosphonates relieve bone pain in this population of patients (27, 28). In the case of bisphosphonates, however, it is unknown if this effect is due to inhibiting osteoclastic activity or due to a direct tumor effect (29, 30).

The C4-2B prostate cancer cells have been reported previously to induce marked osteoblastic skeletal lesions (8, 9). In the current study, we observed osteoblastic areas on radiographs and histology; however, osteolytic lesions were predominant. We cannot readily account for

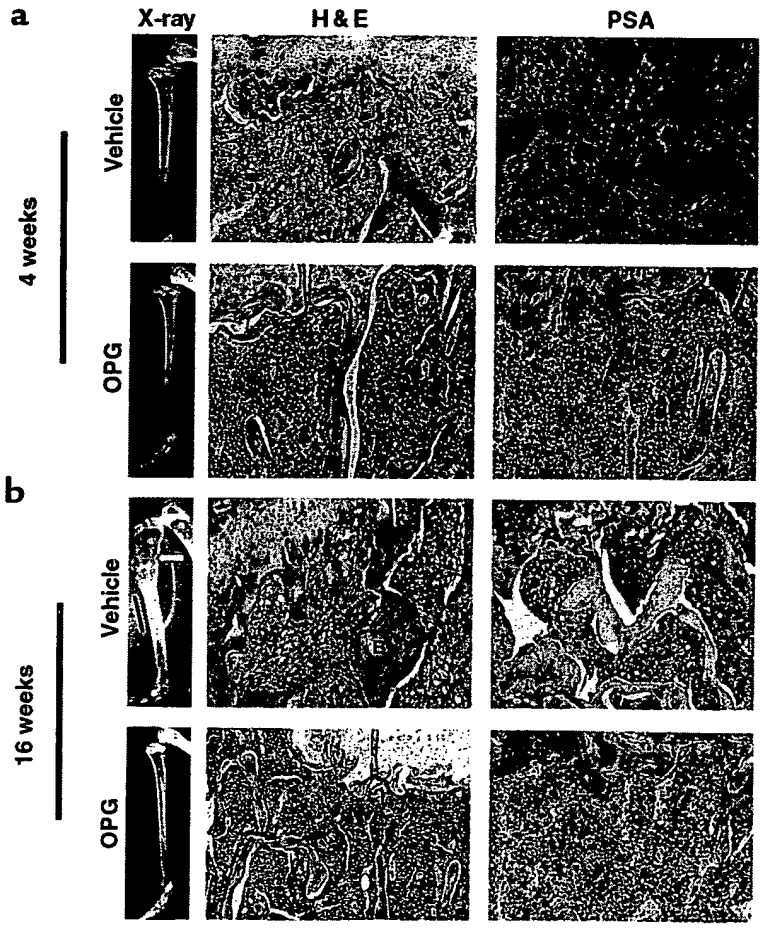

Figure 4
Characteristics of C4-2B bone lesions. SCID mice were injected intratibially with C4-2B prostate cancer cells. At the time of tumor injection, OPG (2 mg/kg) or vehicle (1% BSA in 1× PBS) was administered via the tail vein twice a week for 4 weeks. The mice were sacrificed at 4 weeks and 16 weeks after-tumor injection. Formalin-fixed paraffin-embedded sections were stained with hematoxylin and eosin (H&E) or were deparaffinized, rehydrated, and stained for PSA using immunohistochemistry. Brown coloration indicates presence of PSA. ×200. (**a**) Representative radiographs of H&E- and PSA-stained sections of vehicle-treated versus OPG-treated mice at the end of 4 weeks. Note replacement of bone marrow by tumor in the vehicle-treated animals compared with normal marrow in OPG-treated animals. PSA staining cannot be identified in the OPG-treated animals. (**b**) Representative radiographs of H&E- and PSA-stained sections of vehicle-treated versus OPG-treated mice at the end of 16 weeks. Note the area of osteolysis (arrowhead) and osteoblastic lesion (bar) in the radiograph of the vehicle-treated mouse compared with the normal radiograph of the OPG-treated mice. Also, note the replacement of bone marrow by tumor and the thickened trabeculum indicated with letter B in the vehicle-treated mouse compared with the OPG-treated mouse.

Figure 5
C4-2B cells promote osteoclast activity at bone/tumor interface. SCID mice were injected intratibially with C4-2B prostate cancer cells. Tibias were harvested 16 weeks after tumor injection, decalcified, sectioned, and stained for TRAP. A section is shown that demonstrates multiple TRAP-positive staining osteoclasts at the bone/tumor interface. T, tumor cell; OC, multinucleated TRAP-positive osteoclast. ×1000 under oil.

the discrepancy with previous reports. However, in those studies, tumors were observed in femur, vertebrae, or pelvis (31), in contrast to tibia, as in the current study. Thus, one possibility to account for different degrees of osteoblastic response is the difference in bone remodeling that occurs at different skeletal regions (32–34).

Our observations that C4-2B CM induced osteoclastogenesis from RAW 244.7 cells in the absence of supporting stroma or M-CSF strongly suggested that the CM contained RANKL activity (35). This was further confirmed by the observations that OPG diminished prostate cancer cell CM-induced osteoclastogenesis in RAW 264.7 cells in the absence of supporting marrow stroma and that the CM contained sRANKL. These results suggest that prostate cancer cells directly contribute to osteolysis in vivo through induction of osteoclastogenesis at the metastatic tumor sites and are consistent with reports of the presence of sRANKL in several other cancer cell lines and activated T cells (14–18). In these reports, sRANKL was produced through either proteolytic cleavage of the extracellular portion of RANKL (15, 16) or from an mRNA that encoded a secreted form of RANKL (17). Thus, it is plausible that prostate cancer cells, through their production of proteolytic enzymes such as PSA or metalloproteases (36, 37), cleave the extracellular domain of RANKL, resulting in sRANKL production. Furthermore, the low levels of metalloproteases in SaOS cells may account for our inability to detect sRANKL in the SaOS CM (38).

Our observation that prostate cancer cells express RANKL and directly induce osteoclastogenesis contrasts with reports that an osteolytic murine melanoma and several human breast cancer cell lines do not express RANKL (39, 40). In terms of the murine melanoma cells, RANKL expression was induced in cocultures of melanoma and bone marrow cells (40). However, the source of RANKL was not identified in that study. In contrast, breast cancer cells indirectly induced osteoclastogenesis through upregulation of RANKL in bone marrow stroma and osteoblasts (39). Thus, our results provide a novel mechanism through which discrete osteolytic bone lesions are produced directly by tumor cell–derived sRANKL. This finding is in agreement with the recent report of an mRNA encoding a sRANKL in squamous cell carcinoma cell lines derived from parental malignant tissues that was associated with severe humoral hypercalcemia (17). It is not clear why prostate cancer cell lines express RANKL and breast cancer cell lines do not. One possible explanation accounting for this difference is that as prostate cancer cells progress to a skeletal metastatic phenotype, they take on osteoblast-like characteristics, including production of osteoblast proteins such as bone sialoprotein and osteonectin, expression of the osteoblast-specific transcription factor, Cbfa1, and the ability to form hydroxyapatite in vitro (41, 42). It follows that expression of RANKL, which is expressed in osteoblast, may be upregulated as part of this general phenomenon.

The observation that OPG-mediated prevention of intratibial tumor growth was associated with diminished osteoclastogenesis in vivo, as determined by bone histomorphometry, is consistent with the hypothesis that osteoclast activity is required for establishment of the prostate cancer in bone. In addition to inhibiting osteoclastogenesis, it is possible that OPG directly impaired prostate tumor growth in parallel with its antiosteoclastogenic effect. For example, RANKL is required to prevent apoptosis of epithelial mammary cells through interaction with RANK on the mammary cell surface

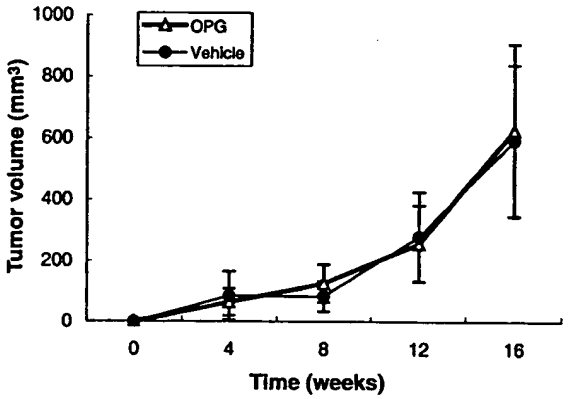

Figure 6
OPG does not affect subcutaneous tumor growth in mice. C4-2B prostate cancer cells were injected subcutaneously into the SCID mice at the same time they received intratibial tumor injection as described in Figure 4. At the time of tumor injection, OPG (2 mg/kg) or vehicle (1% BSA in 1× PBS) was administered via the tail vein twice a week for 4 weeks. Tumors were allowed to grow for another 12 weeks. The mice were sacrificed at 16 weeks after tumor injection. Tumor volume was measured monthly. Data are reported as mean (±SD) from nine to ten animals per group.

during development (43). This observation raises the possibility that OPG, which blocks RANKL activity, may induce apoptosis of other epithelial cell types, including prostate cells. However, based on the observations that (1) direct administration of OPG had no detectable effect on prostate cancer cell proliferation, viability or apoptotic rate in vitro and (2) OPG did not impair prostate tumor growth in the subcutaneous site, it is unlikely that OPG mediated its tumor growth preventative effects through a similar mechanism. Taken together, these data provide support to the possibility that OPG prevents prostate tumor establishment in bone through inhibition of osteoclastogenesis in this animal model. However, the current experiments do not completely rule out that OPG enhanced the expression of factors that inhibit tumor growth specifically in the bone microenvironment.

Our result that OPG prevented both osteolysis and establishment of tumor is in agreement with a previous report that LNCaP cells implanted in the femurs of nude mice initially formed tumor in bone, which spontaneously regressed to be replaced by normal marrow (44). However, our results present an interesting contrast to several studies that have examined the effect of inhibiting osteoclastic activity on tumor development in mice. In one study, ibandronate prevented myeloma-associated osteoclastogenesis (45), and in another study OPG diminished the osteolytic component of a primary sarcoma implanted in the bone of mice (7); but in contrast to our study, tumor volume was not decreased compared with control animals in either study. Possible reasons for this difference between the current study and these previous reports may be because bone was the myeloma's and sarcoma's primary site, thus the tumor was in a favorable environment and may readily thrive in the bone microenvironment. In contrast, prostate cancer is an epithelial tissue derived from a site other than bone, and thus the prostate cells are in a "hostile" environment as proposed previously (41). Furthermore, it has been suggested that the bone resorption releases growth factors from the bone matrix that promote tumor growth (24). Thus, the prostate cancer cells may not thrive in the hostile bone microenvironment in the presence of osteoclast activity and the resulting release of growth factors.

In summary, results from the current study demonstrated that prostate cancer cells produce sRANKL and induce osteoclastogenesis in vitro. Furthermore, it demonstrated that OPG prevents establishment of prostate cancer in bone, but not in subcutaneous tissue. Taken together, these results suggest the osteoclast activity is an important component of the establishment of prostate cancer in the skeleton and that inhibition of osteoclastic activity may prevent establishment or slow progression of skeletal metastatic lesions, including those with an osteoblastic component.

Acknowledgments

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